DNA Vaccination Against *Entamoeba histolytica*

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ABSTRACT

Invasive amebiasis, caused by the protozoan parasite *Entamoeba histolytica*, is one of the leading parasitic causes of mortality worldwide, and there are no vaccines available to control the disease. The heavy subunit of the *E. histolytica* Gal-lectin is regarded as a potential subunit vaccine candidate. A Th1 (cell-mediated) immune response is protective against invasive amebiasis, and DNA vaccination is a strategy to induce such a response against specific antigens. The objective of this study was to construct and test a Gal-lectin-based DNA vaccine against *E. histolytica*. DNA encoding aa 894–1081 of the Gal-lectin heavy subunit was resynthesized using a gerbil codon frequency bias and inserted in a mammalian expression vector to generate the DNA vaccine pCISToGL6. Balb/c mice vaccinated intradermally developed a Gal-lectin-specific cellular immune response, as well as an anti-Gal-lectin humoral immune response. Serum antibodies recognized a recombinant portion of the Gal-lectin heavy subunit by immunoblot and ELISA, and bound to native Gal-lectin on the surface of live trophozoites, inhibiting adherence to target cells. The Gal-lectin-specific serum antibodies were of the IgG2a isotype, indicating that a Th1 response was stimulated by the vaccine. We were also interested in using DNA encoding IL-12, IL-18 or GM-CSF as genetic adjuvants co-injected with pCISToGL6 to potentiate the immune response. Since the DNA vaccine was destined to confer protection in the gerbil model of invasive amebiasis, we cloned gerbil IL-12 (p35 and p40), IL-18 and its convertase caspase-1, and GM-CSF. The proteins were expressed in mammalian cells and showed bioactivity *in vitro*. Taken together, these results have laid the foundation to optimize and test a working Gal-lectin-

with co-stimulatory molecules to elicit a Th1 immune response for protective immunity against invasive amebiasis.

ABRÉGÉ

L'amibiase invasive, qui est causée par le protozoaire *Entamoeba histolytica*, est l'une des principales causes de mortalité d'origine parasitaire au monde, et il n'existe aucun vaccin pour contrôler la maladie. La sous-unité lourde de la Gal-lectine d'*E. histolytica* est reconnue comme étant un vaccin potentiel. Une réponse immunitaire de type Th1 (immunité cellulaire) protège contre l'amibiase invasive, et la vaccination par ADN est un moyen d'induire une telle réponse contre un antigène spécifique. L'objectif de cette étude était donc de construire et de tester un vaccin à l'ADN codant la Gal-lectine ou une de ses portions, et étant efficace contre *E. histolytica*. L'ADN encodant les acides aminés 894–1081 de la sous-unité lourde de la Gal-lectine a été re-synthétisé en employant un biais de fréquence de codons propre aux gerboises, et a été inséré dans un vecteur d'expression de cellules de mammifères, afin de générer le vaccin à l'ADN pCISToGL6. Des souris Balb/c vaccinées de façon intradermale ont développé une réponse immunitaire cellulaire spécifique à la Gal-lectine, ainsi qu'une réponse immunitaire humorale contre la Gal-lectine. Les anticorps du sérum des animaux vaccinés ont reconnu une portion recombinante de la sous-unité lourde de la Gal-lectine par les techniques immunoblot et ELISA, et se sont fixés à la Gal-lectine native à la surface de trophozoites vivants, bloquant ainsi leur adhérence aux cellules cibles. Ces anticorps spécifiques à la Gal-lectine étaient d'isotype IgG2a, ce qui suggère que le vaccin a stimulé une réponse de type Th1. Nous étions aussi intéressés à utiliser l'ADN encodant l'IL-12, IL-18, ou GM-CSF, comme adjuvant génétique co-injecté avec pCISToGL6, afin d'augmenter la réaction immunitaire. Puisque le vaccin était destiné à être utilisé chez la

gerboise, nous avons cloné les ADNc de l'IL-12 (sous-unités p35 et p40), IL-18, sa convertase caspase-1 et GM-CSF. Nous avons exprimé les protéines dans des cellules de mammifères, et nous avons démontré leur bioactivité *in vitro*. Ces résultats représentent les premiers pas vers l'optimisation et le développement d'une vaccination à l'ADN basée sur la Gal-lectine et couplée de molécules co-stimulatrices, afin d'éliciter une réponse immunitaire protectrice de type Th1 pour lutter contre l'amibiase invasive.

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LIST OF ABBREVIATIONS

aa	amino acid
Ag	antigen
ALA	amebic liver abscess
APC	antigen-presenting cell
BCG	Bacille Calmette Guérin
BGH	bovine growth hormone
BHK	baby hamster kidney
bp	base pair(s)
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium level
cDNA	complementary DNA
CHO	Chinese hamster ovary
CMI	cell-mediated immunity
ConA	concanavalin A
COX	cyclooxygenase
CpG	cytosine-phosphate-guanine
cpm	counts per minute
CR	cysteine-rich region
CRD	carbohydrate recognition domain
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
ECL	enhanced chemoluminescence
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
Gal	galactose
Gal-lectin	galactose-inhibitable adherence lectin
GalNAc	N-acetyl galactosamine
GM-CSF	granulocyte/macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
GROα	growth-regulated oncogene alpha
GST	glutathione S-transferase
h	hour(s)
H_2O_2	hydrogen peroxide
HA	hyaluronic acid
hCMV	human cytomegalovirus
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin

iNOS	inducible nitric oxide synthase
kDa	kilodalton(s)
LB	Luria-Bertani
LPG	lipophosphoglycan
LPS	lipopolysaccharide
MAb	monoclonal antibody
MBP	maltose-binding protein
MDCK	Madin-Darby canine kidney
MHC	major histocompatibility complex
MW	molecular weight
mRNA	messenger ribonucleic acid
NK	natural killer
NO	nitric oxide
nt	nucleotide(s)
ORF	open reading frame
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEC	peritoneal exudate cell
PG	prostaglandin
PHA	phytohaemagglutinin
PR	pseudorepeat
RACE	rapid amplification of cDNA ends
RT	reverse transcription
SAA	soluble amebic antigen
SCID	severely combined immunodeficient
SCID-HU-INT	SCID-human intestine
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	standard error
sIgA	secretory immunoglobulin A
SV40	simian virus 40
SREHP	serine-rich *E. histolytica* protein
TGF	transforming growth factor
TNF	tumor necrosis factor
UTR	untranslated region

THESIS OFFICE STATEMENT

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory**.

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections**: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) final overall conclusion and/or summary.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (*eg.* in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent**; supervisors must attest to the accuracy of such claims at the

Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

STATEMENT OF ORIGINALITY

The following aspects described in this thesis are considered contributions to original knowledge:

MANUSCRIPT I

This is the first report of an immunologically relevant portion of the Gal-lectin administered as a DNA vaccine and inducing an anti-amebic immune response. Furthermore, we enhanced the expression of an *E. histolytica* protein in mammalian cells by re-writing the DNA coding for it, using a mammalian codon usage frequency. To our knowledge, this had never been done before.

MANUSCRIPT II

The Mongolian gerbil, *Meriones unguiculatus*, is permissive to several infectious agents, making it suitable for use as an animal model for those diseases. However, immunological studies using this animal have been hampered due to a lack of gerbil-specific immunological reagents. This is the first report of molecular cloning, expression and characterization of gerbil IL-12.

MANUSCRIPT III

We were the first to clone the cDNAs coding for gerbil IL-18 and its convertase, caspase-1, and to show bioactivity of the molecules when expressed in mammalian cells *in vitro*.

MANUSCRIPT IV

This is the first report of the cloning, *in vitro* expression and characterization of GM-CSF from the Mongolian gerbil.

STATEMENT OF AUTHORSHIP

The experiments described in Manuscripts I-IV were all designed and carried out by myself. I am also responsible for the data analysis and manuscript preparation. Dr. Kris Chadee, the only co-author in all the manuscripts, acted as thesis supervisor. Dr. Chadee provided financial resources for the laboratory work and advice on experimental design, data analysis and preparation of the manuscripts and thesis.

SECTION I: LITERATURE REVIEW

INTRODUCTION

The intestinal protozoan parasite *Entamoeba histolytica* was named by Schaudinn in 1903 after the organism's outstanding ability to destroy human tissues (1). It is estimated that one percent of the world's population shows clinical signs resulting from infection with the pathogen, ranging from mild intestinal ulcerations to amebic liver abscess due to metastatic spread of the parasite. In spite of the existence of effective antiamebic chemotherapy, amebiasis is the third leading parasitic cause of mortality in the world.

Despite the deployment of anti-amebic innate and immune responses by the host, amebae may still survive due to the development of resistance mechanisms and the modulation of the host's immune response. Amebic cysteine proteinases are able to degrade serum and secretory antibodies (2, 3), while the galactose-inhibitable adherence lectin (Gal-lectin) confers resistance to complement-mediated lysis (4). *E. histolytica* suppresses T lymphocyte responsiveness (5, 6) and macrophage functions, such as locomotion, respiratory burst (7, 8), nitric oxide production (9) and major histocompatibility complex class II molecule expression (10), by means of secreted products and/or stimulation of prostaglandin production by the host. Meanwhile, virulence factors elaborated by the parasite, such as cysteine proteinases, amebapores, and the Gal-lectin, allow trophozoites to cause tissue injury (11, 12), ultimately resulting in the death of the host if the infection is left untreated.

Laboratory animals experimentally infected with *E. histolytica* develop resistance to a challenge infection upon cure with metronidazole (13), which implies that protective

immunity to amebiasis can be achieved. In addition, recent epidemiological data show a correlation between presence of anti-amebic coproantibodies and resistance to reinfection (14), suggesting that protective immunity also happens in humans and that an amebiasis vaccine could be possible.

The Gal-lectin is a multi-purpose glycoprotein that allows trophozoites to resist complement lysis, and to adhere to colonic mucins for colonization and to target cells for tissue destruction (15, 16). On the other hand, it is also a molecule of choice to be included in an amebiasis vaccine. Numerous immunization studies published to date have reported the great vaccine potential of the native molecule or portions of its 170 kDa heavy subunit. These proteins comprise T and B cell epitopes and, when injected in laboratory animals, stimulate Gal-lectin-specific T cells (17) as well as antibodies that can inhibit adherence of trophozoites to target cells (18). The Gal-lectin also stimulates production of the proinflammatory cytokines interleukin-12 and tumor necrosis factor by macrophages (19, 20), thereby acting as its own Th1 adjuvant. A Th1 (cell-mediated) immune response is believed to be protective against *E. histolytica*, as the cytokines produced, especially interferon-γ, activate macrophages to produce the amebicidal molecule nitric oxide (21, 22).

DNA vaccination is an increasingly popular approach to induce a cell-mediated immune response against a specific antigen, and has been used successfully to confer protection against a variety of pathogens. The mechanism behind this strategy is simple; plasmid DNA containing a sequence encoding an antigen (or an immunologically relevant portion thereof) is produced in and purified from *Escherichia coli* and injected in the subject, either intramuscularly or intradermally. The host cells get transfected *in vivo*

by the plasmid and express the encoded antigen against which the host's immune system mounts a response (23). Specific nucleotide sequences found in the plasmid, called CpG motifs, have immunostimulatory properties, promoting, among other things, the production of TNF and IL-12 by macrophages (24), and therefore acting as strong Th1 adjuvants. Co-injection of the DNA vaccine with plasmid DNA encoding certain cytokines or co-stimulatory molecules can further influence the amplitude and/or polarization of the immune response to the antigen (25).

The objective of this study was to develop a Gal-lectin-based DNA vaccine against *E. histolytica* to be used in the gerbil model of amebiasis. The first specific aim was to identify an immunologically relevant portion of the Gal-lectin, to include it in the vaccine and to test the construct in mice in order to assess its immunogenicity. For further enhancement of the efficacy of the vaccine, and to ensure that a strong Th1 response is induced by it, we proposed to use cytokine and accessory molecule genes as adjuvants. However, since the DNA vaccine was ultimately destined to be used in gerbils, and since only a few DNA sequences encoding gerbil cytokines have been determined so far, the second specific aim of this work was to clone the genes encoding gerbil interleukin-12, granulocyte/macrophage colony-stimulating factor and interleukin-18, and express and characterize the molecules.

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CHAPTER 1. INVASIVE AMEBIASIS

1.1 *E. histolytica* Life Cycle

The life cycle of *E. histolytica* is fairly simple (1). Food or water contaminated with feces from infected people and containing the infective cysts (8-20 μm) is first ingested by the host. The cysts, which are resistant to the acidic environment of the stomach, reach the small and large intestines where they excyst, giving rise to eight motile, invasive trophozoites (10-60 μm). The parasites then multiply by binary fission, feeding on the gut flora, to ultimately form cysts that are evacuated with the feces and contaminate the environment to continue the cycle. Occasionally, amebae penetrate the gut mucosa and disseminate to other organs, causing intestinal and extraintestinal amebiasis, respectively.

1.2 Epidemiology of Amebiasis

There are no vectors involved in the transmission of amebiasis, and humans and some primates are the only hosts for the parasite. *E. histolytica* has a worldwide distribution but causes the highest mortality in Africa, South America, India and Mexico, where hygienic practices are not optimal. In other areas, high risk individuals are emigrants from endemic areas (2), travelers (3), sexually active homosexual men (4-6) and residents of mental institutions (7-9). Amebic liver abscesses are more common in people between 20 and 40 years of age (10, 11), but young children, pregnant women and elderly people experience the most severe cases (12, 13). There is a striking

preponderance of males among patients with ALA (ratios of about 10 males:1 female) (14) and it has been suggested that alcohol use may explain this phenomenon (15).

It was previously thought that *E. histolytica* infected about 480 million individuals annually, worldwide, but about a tenth of these cases progressed to symptomatic infections (amebic colitis and liver abscess), ultimately resulting in 40,000 to 110,000 deaths (12). It was later realized that most asymptomatic infections were due to "nonpathogenic" amebae that are morphologically very similar to the "pathogenic" ones that cause disease, but that can be clearly and reliably differentiated immunologically using monoclonal antibodies (MAbs) directed against certain surface or intracellular amebic molecules (16-21), or genetically using specific DNA probes (22-25), polymerase chain reaction (26), or restriction fragment length polymorphism (27). *Entamoeba histolytica* was thus re-described; the "non-pathogenic" species was termed *E. dispar*, while the "pathogenic" amebae retained the name *E. histolytica* (28). *E. histolytica* and *E. dispar* are indeed two different species, and the genetic distance between them has been estimated to be as great as that between humans and mice (29).

It must be noted that the words "pathogenic" and "nonpathogenic" have been extensively misused in the amebiasis literature, often confusing them with "virulent" and "avirulent", respectively, when referring to the amebae causing (or not) the disease. An article was finally published in 1994, clarifying this situation (30). The authors reminded the readers of the true definition of "pathogenicity", which is the general ability of an organism to cause disease (a given organism is therefore either a pathogen or not), and of "virulence", which is the relative capacity of an organism to cause disease in a specific condition (a pathogen can be of varying virulence, depending on the circumstances, such

as the genetic makeup of the host or its state of immunosuppression). Although *E. histolytica* often causes asymptomatic infections, it is always pathogenic but with different degrees of virulence. While *E. dispar* infections are always asymptomatic, it was found that the organism is still capable of causing focal lesions by erosion of the colonic mucosa (6, 31, 32). *E. dispar* thus has to be classified as an avirulent, non-invasive pathogen (30).

1.3 Pathogenesis of Amebiasis

The pathogenesis of *E. histolytica* occurs in four stages. The first stage, the colonization of the mucosal surface of the bowel by trophozoites, starts when the parasite binds to mucin molecules of the mucus layer *via* the membrane-associated galactose-inhibitable adherence lectin (Gal-lectin) (33, 34). Mucins, due to their gel-forming properties and highly heterogeneous oligosaccharide composition, prevent amebae from adhering to and destroying the underlying epithelial cells (33, 35-37). The intestinal flora also competes with amebae for attachment to mucin molecules and intestinal colonization (38).

The second stage is the depletion and dissolution of the mucus layer. Trophozoites produce a potent mucus secretagogue with an activity comparable to that of cholera toxin (39, 40), that stimulates rapid, protein kinase C-dependent hypersecretion of preformed mucin stores and newly synthesized mucin from intestinal goblet cells (41). This results in the depletion of the protective mucus blanket, eventually facilitating amebic invasion. Such an effect is also seen in experimental amebiasis studies, where amebic invasion of

the colonic mucosa is always preceded by the depletion of luminal and goblet cell mucins (42, 43).

Amebae can also degrade the mucus layer by means of cysteine proteinases (CPs) (44, 45) and possibly glycosidases. To date, 7 genes encoding CPs have been identified in the *E. histolytica* genome (46); however, most of the CP activity in trophozoite lysates results from the product of 4 of them: EhCP1, EhCP2, EhCP3 and especially EhCP5 (47, 48). It has recently been shown in our laboratory that products secreted by *E. histolytica* trophozoites degrade purified intestinal mucins, and that this effect can be inhibited by the cysteine proteinase inhibitor E-64 (44). Expression of glycosidases is common in mucin-dwelling protozoans (49), and β-N-acetylgalactosaminidase and α-mannosidase activities have been detected in *E. histolytica* (49, 50). Trophozoites may potentially use them, in concert with CPs, to degrade mucin molecules and decrease their protective effect (44). On the other hand, it was suggested that gut flora-derived glycosidases, together with host proteases found in the intestine, can decrease adherence of trophozoites to target cells by degrading the Gal-lectin (51).

The third stage of pathogenesis involves the attachment of amebae to intestinal epithelial cells, cytolysis and tissue destruction. As for intestinal mucin, adherence of trophozoites to target cells is also mediated mainly by the Gal-lectin; galactose (Gal), N-acetyl galactosamine (GalNAc), anti-Gal-lectin MAbs and secretory IgA (sIgA) all inhibit this phenomenon (52-54). As discussed below, cytolysis of these cells is dependent upon this adherence. However, the damage to the mucosa is not only due to host cell lysis by amebae. When in contact with intestinal epithelial cell monolayers, trophozoites cause disruption of tight junctions between cells, leading to increased intestinal permeability

(55-57). Amebae can also degrade the extracellular matrix by means of proteases (58), causing rounding and detachment of the cells; CPs seem to be mainly responsible for this effect (59). It has been demonstrated that CPs degrade laminin (60). Trophozoites (or their extract) overexpressing EhCP2 showed an enhanced ability to cause Chinese hamster ovary (CHO) cell monolayer destruction, and this was blocked by the addition of E-64 (61). Curiously, inhibition of CP expression by antisense RNA to EhCP5 (90% decrease in total CP activity) did not reduce the ability of trophozoites to destroy BHK monolayers (62). However, addition of E-64 to the trophozoites completely (63) or partially (61, 62) decreased the cytopathic effect, clearly demonstrating a role for CPs. Collagenase contained within electron-dense granules in trophozoites could also act on the extracellular matrix to degrade it (64, 65).

Cell and tissue destruction can also be due to infiltration and lysis of inflammatory cells, such as neutrophils, at the site of invasion. As amebic invasion progresses in the lamina propria and the crypts, neutrophils (and to a lower extent lymphocytes and eosinophils) are recruited to these sites (43). Ameba trophozoites produce a chemoattractant for neutrophils (66, 67). Co-culture of amebae with intestinal epithelial cells stimulates the production of a variety of proinflammatory and chemoattractant cytokines, such as interleukin (IL)-8, GROα, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-1α and IL-6 (68-71), which also attract neutrophils to the site of invasion. While it has been shown in our laboratory that medium conditioned by live trophozoites can induce IL-8 gene expression and protein production by the cells (69), others have reported that induction of the protein requires ameba-target cell contact inhibitable by Gal or GalNAc (68, 70). It is possible that trophozoites lyse some epithelial

cells, which then release preformed IL-1α (68) or pro IL-1β that gets activated by amebic cysteine proteinases with IL-1β converting enzyme activity (72). This processed IL-1 would then act on neighboring intact cells, inducing them to produce IL-8. Amebic infection in the gut also stimulates cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) production by epithelial cells and macrophages (73, 74), presumably through the action of IL-1, and it has been shown that PGE_2 stimulates IL-8 expression in colonic epithelial cells (75).

Amebae are very cytotoxic to unactivated neutrophils (76). Upon contact with trophozoites at the site of recruitment, neutrophils lyse, releasing their toxic content into their surroundings (proteases, cathepsins, lysozymes), killing nearby cells and worsening intestinal damage (77, 78). The reduction of gut tissue destruction by depleting neutrophils from SCID-HU-INT mice that had their human intestinal xenograft infected with *E. histolytica* trophozoites clearly demonstrates the role of these inflammatory cells in amebic ulceration of the colonic mucosa (79).

The fourth stage of pathogenesis in amebiasis is the dissemination of amebae to internal soft organs. Once the trophozoites reach the blood vessels of the intestine, they penetrate into them and spread hematogeneously to the liver, and in some cases to the spleen or even the brain. Amebic CPs may play an important role in the pathogenesis of ALA; CP-deficient amebae, which were defective in phagocytosis, were unable to cause liver lesions in an animal model (80), and pretreatment of virulent trophozoites with E-64 abrogated or reduced their ability to cause ALA in SCID mice (81). However, overexpression of EhCP2 in amebae did not augment their ALA formation potential (61). Perhaps this specific CP plays a minor role in the pathogenesis of ALA compared to other

amebic CPs, such as EhCP5. The Gal-lectin and amebapores (see below) are two other important factors in pathogenesis, as inhibition of their expression using antisense technology decreased the ability of amebae to cause ALA in animals (82, 83).

As it is the case for intestinal amebic lesions, ALA formation is mainly due to the lysis of recruited inflammatory cells by trophozoites and the release of enzymes from them (84), causing extensive necrosis of the surrounding tissue. Abscesses increase in size as more necrosis occurs and contain purulent, acellular proteinaceous debris surrounded by a rim of trophozoites at the edges (85) and a thin wall of connective tissue (86). In humans, the infection can be lethal if left untreated.

It is now recognized that the cytolytic activity of *E. histolytica* is dependent on Gal-lectin-mediated amebic adherence to the target cell; if this contact is blocked by means of Gal, GalNAc or adherence-inhibitory anti-Gal-lectin MAbs, the ability of the parasite to lyse target cells is abrogated (53, 87, 88). CHO cells deficient in N-acetylglucosaminyltransferase I, which have decreased N-linked N-acetyl lactosamine levels on their surface, are resistant to killing by trophozoites (89). Seconds after contact has been made, there is a 20-fold, rapid and irreversible increase in intracellular calcium level ($[Ca^{2+}]_i$) in the target cell, which is associated with target cell membrane blebbing. Cell death occurs 5 to 15 minutes later (90). It has recently been proposed that this rise in $[Ca^{2+}]_i$ could act as a second messenger that triggers signal transduction pathways leading to cell death, as activation of the protein tyrosine phosphatase PTP1B by calpain and protein dephosphorylation were observed in Jurkat cells exposed to live trophozoites prior to their death (91).

Whether target cells die *via* necrosis or apoptosis is somewhat controversial. While one laboratory reported that the human leukemic cell lines HL-60 and Jurkat undergo necrosis (characterized by cell swelling, rupture of the plasma membrane and release of cellular contents) when incubated with live trophozoites (92), studies from other teams report a non-classical apoptotic mechanism of cell death. It was initially reported that target cells undergo DNA fragmentation (characteristic of apoptosis) which can not be blocked by overexpression of Bcl-2, a protein that inhibits apoptotic cell death induced by some stimuli (93). Apoptosis caused by *E. histolytica* occurs in a Fas- and tumor necrosis factor (TNF)-independent fashion, as demonstrated by the fact that hepatocytes in mice that are deficient in Fas, Fas ligand, or TNF receptor I still undergo apoptosis when the animals are injected in the liver with live trophozoites (94). Using the SCID mouse model of ALA, it was shown that *in vivo* blockade of caspases with the caspase inhibitor Z-VAD-FMK could partially inhibit ALA formation and completely block apoptotic cell death in the abscesses (95). *In vitro*, Jurkat cells exposed to live trophozoites undergo apoptosis which is preceded by the activation of caspase-3, independent of caspases 8 and 9, which are upstream in the caspase cascade (96). It is not known whether this caspase-3 activation is due to a host or amebic protease. As mentioned above, calpain may play a role, since it is activated in target cells upon contact with trophozoites (91), and it has been reported to promote apoptosis in some systems (97).

This "lethal hit" delivered to the target cell after amebic adherence is believed to be mediated by amebapores (98-102), which are membrane pore-forming peptides secreted by amebae upon contact with host cells (103). However, it has been shown that

purified amebapores cause necrotic cell death rather than apoptosis (92), suggesting that, while they may play an important role in host cell killing, they may not be the only effector molecules.

A few other amebic molecules with cytolytic or cytopathic activities have been described. Hemolysins, encoded on an extrachromosomal element also coding for ribosomal RNA, are cytotoxic to human colonic epithelial cells (104). Fifteen genes encoding saposin-like proteins different from amebapores are present and transcribed in *E. histolytica* trophozoites; the peptides they encode still await characterization (105). Amebic phospholipase A (106) activity is required for cytolysis of target cells, as pharmacological antagonists of eukaryotic phospholipase A inhibited killing of CHO cells (107). Acid optimal lysosomal enzymes produced by amebae seem also to play a role in host cell killing (108). Finally, the Gal-lectin itself may play a role in cytopathogenesis distinct from its adherence function; it was found that an anti-Gal-lectin MAb could block cytotoxicity of *E. histolytica* trophozoites after adherence to a target cell had occurred (88). It was suggested that the antibody may interfere with the ability of the lectin to transduce a signal to the cytoplasm that initiates cytolysis.

1.4 The Gal-lectin

The Gal-lectin of *E. histolytica* is the major surface molecule involved in amebic adherence to colonic mucin, colonic epithelial cells, macrophages, neutrophils, peripheral blood lymphocytes, red blood cells and bacteria (33, 53, 109-112). This lectin has a carbohydrate specificity for Gal and GalNAc, as the two simple sugars inhibit adherence of trophozoites to the colonic mucosa (113) and to target cells (53). Initial studies on the

characterization of the target cell carbohydrate receptors for the Gal-lectin, involving a panel of CHO glycosylation mutants, revealed that the Gal-lectin binds preferentially to β1-6-branched, N-linked carbohydrates lacking terminal sialic acid or fucose residues (114). Later, it was found that the Gal-lectin has a higher affinity for complex, branched carbohydrate chains with Gal or GalNAc nonreducing terminal residues, as they were more effective, by weight, at inhibiting amebic adherence than the free sugars (115). More recent studies on the sugar binding specificity of the Gal-lectin revealed that the latter binds with very high affinity to glycoproteins containing polyvalent N-acetylgalactosaminides (116), more specifically to those with GalNAc-terminal oligosaccharide chains that are loosely clustered, as they are found in mucin molecules (117). In fact, the Gal-lectin has a very high affinity for rat and human colonic mucins; the purified molecules were 10 000 fold more effective by weight than Gal or GalNAc monomers at inhibiting adherence (33). Binding of the Gal-lectin to carbohydrates is a calcium-dependent event (116).

Some MAbs raised against the native Gal-lectin have been shown to inhibit adherence of trophozoites to CHO cells and human colonic mucin (52, 118), while others enhanced it (119). An explanation for this could be that binding of these antibodies may promote a conformational change in the lectin's structure, decreasing or augmenting its affinity for its carbohydrate ligand. The lectin would thus be conformationally flexible, and could switch back and forth between ligand high- and low-affinity states of adhesion, allowing for locomotion on the substrate, as it is thought to happen with integrins in leukocytes (120). In fact, the Gal-lectin was found to share some structural properties with integrins (see below). It is believed that amebae shed a part of their Gal-lectin pool

in the process of tissue invasion, as the molecules were detected bound to the lateral surface of intestinal cells when the latter were co-cultured with live trophozoites (121). It has been suggested that the Gal-lectin may also play a role during encystation, by sensing the concentrations of available Gal and GalNAc in the gut environment and promoting ameba aggregation that precedes encystation (122).

The Gal-lectin is a heterodimeric, 260-kDa glycoprotein consisting of a heavy subunit of 170 kDa and a 31/35-kDa light subunit linked by disulfide bonds in a 1:1 molar ratio (Fig. 1) (123). Heavy and light subunit monomers are not found in significant quantities (124). It is larger than other eukaryotic Gal-binding lectins (125, 126) but its size and complexity are similar to other cell-adhesion molecules such as the leukocyte adhesion receptor Mo1 (127) and the neural and liver cell adhesion molecule (128). The heavy and light subunits have different amino acid sequences, are structurally dissimilar and show no antigenic cross-reactivity (129-131). The Gal-lectin itself, on the basis of its structure, cannot be classified within any of the known kinds of lectins found in higher eukaryotes.

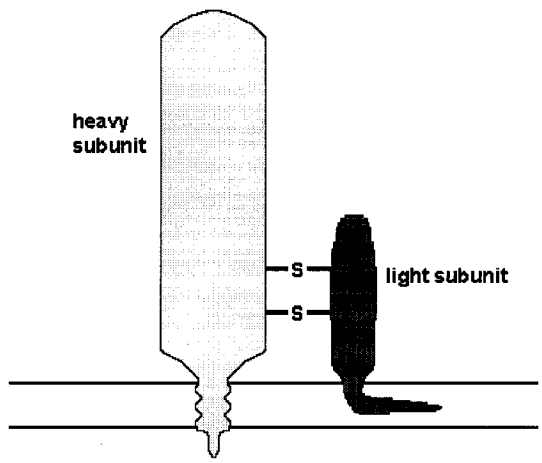

Figure 1. Schematic representation of heterodimeric Gal-lectin.

1.4.1 The Heavy Subunit

The 170-kDa heavy subunit of the Gal-lectin is encoded by the *hgl* gene family, which includes at least five members (*hgl1-5*) (129, 130, 132, 133). The first three have been completely sequenced and were found to be highly conserved; *hgl1* and *hgl3* are 95.2% identical at the amino acid (aa) sequence level, while they share 89.2% and 89.4% identity, respectively, with *hgl2* (133). The five genes were mapped to five distinct *Hind*III restriction fragments in the *E. histolytica* genome (132). At least three of the *hgl* genes (*hgl1*, *hgl2* and *hgl4*) are expressed simultaneously (132, 133). The promoter sequence of the *hgl5* gene has been extensively studied, and several regulatory elements have been identified within it (134-138). The aa sequence of the mature 170-kDa subunit has been determined (129, 130). It is made up of 1276 residues, which can be grouped into three distinct domains: a 1209-aa extracellular domain, a 26-aa hydrophobic transmembrane domain, and a short carboxy-terminal cytoplasmic tail made up of 41 residues (Fig. 2).

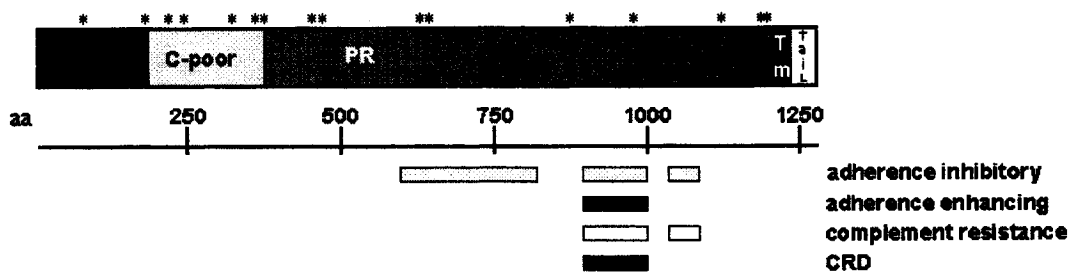

Figure 2. Schematic representation of the heavy subunit of the Gal-lectin. Epitopes recognized by adherence inhibitory, adherence enhancing, and complement resistance blocking MAbs are shown by grey or white boxes. The putative CRD is shown by a black box. Asterisks represent the locations of potential N-linked glycosylation sites.

The cytoplasmic tail of the heavy subunit contains several potential phosphorylation sites (130) and shares sequence identity with the tyrosine autophosphorylation site of the epidermal growth factor receptor, suggesting that it may play a role in transmembrane signaling. The tail region also contains sequence identity with the β2 and β7 integrin cytoplasmic tails, including aa residues involved in control of adhesion. Recently, it was shown that amebae intracellularly expressing a green fluorescent protein fusion protein containing the cytoplasmic tail of the heavy subunit showed a dominant negative effect on lectin-mediated adherence and cytotoxicity to target cells *in vitro* as well as decreased virulence *in vivo*. Mutations in the tail sequence restored virulence to wild-type levels. This indicates that intracellular factors may interact with the cytoplasmic tail and mediate inside-out signaling *via* the Gal-lectin for control of adherence (139).

The large extracellular domain of the heavy subunit is heavily glycosylated; it has 16 potential sites for N-linked glycosylation (Fig. 2), and it was estimated that about 6% of the subunit's apparent molecular mass is due to carbohydrate residues (129). Two different MAbs raised against human β2 integrins recognized epitopes within the 170-kDa subunit, suggesting an explanation for the ability of trophozoites to invade blood vessels and interact with the extracellular matrix in the liver (140). The extracellular domain itself can be subdivided into three regions: a cysteine/tryptophan-rich region (aa 1-187), a cysteine-poor region (aa 187-377), and a cysteine-rich region (CR; aa 378-1208) that includes a pseudorepeat portion (PR; aa 378-653). The CR, which contains 10.8% cysteine residues, is thought to confer resistance to host- and ameba-derived proteases (129). The CR also shows sequence similarities and antigenic cross-reactivity with CD59,

the human membrane inhibitor of the complement membrane attack complex C5b-9 (141).

The epitopes recognized by the MAbs that inhibit or enhance amebic adherence all mapped to the CR (52), suggesting that the CR contains the carbohydrate-recognition domain (CRD) of the lectin. Recently, it has been shown that an *in vitro*-translated protein including aa 352-1139 of the heavy subunit exhibited GalNAc-specific binding activity (142). In another report, a small recombinant protein including aa 895-998 was produced in *E. coli* and was shown to bind GalNAc with high affinity (143), clearly fine-mapping the CRD to that region. It was also suggested that the PR portion also contains a CRD, as a recombinant protein including it induced proliferation of naïve gerbil splenocytes, similar to a plant lectin (144).

1.4.2 The Light Subunit

Upon reducing SDS-PAGE, immunoaffinity-purified Gal-lectin separates into the heavy subunit and two light subunit isoforms of 31 and 35 kDa (123, 131). Two-dimensional gel electrophoresis shows that the two isoforms make distinct 170/31 and 170/35 heterodimers (131). This heterogeneity is most likely due to different post-translational modifications, as the aa compositions of the two isoforms are nearly identical. Microheterogeneity has been found at the amino terminus of the light subunit, suggesting that it is encoded by more than one gene. Southern blot analysis on restriction enzyme-digested *E. histolytica* genomic DNA also supported this hypothesis (131). It is believed that a minimum of six genes code for the light subunit, and at least three of them

are simultaneously expressed (132). To date, three of these genes have been identified and are referred to as *lgl1* (131), *lgl2* (145) and *lgl3* (132).

The function of the light subunit is still unknown. It is not related to any other known kinds of proteins and does not contain any typical CRD (131). MAbs raised against it do not inhibit adherence of trophozoites to target cells, which rules out a direct role for the light subunit in this process (124). The molecule is a glycoprotein, containing two potential N-linked glycosylation sites. It has no transmembrane domain, but contains a hydrophobic carboxyl-terminal domain, which is a characteristic of acyl-glycosylphosphatidylinositol (GPI)-anchored proteins. Indeed, fatty acid incorporation analysis showed that the light subunit is post-translationally modified by the addition of acyl-GPI (131). Deletion of the GPI anchor signal sequence prevents its assembly with the heavy subunit to form the Gal-lectin heterodimer (146). Trophozoites deficient in light subunit expression showed a decrease in cytopathic and cytotoxic activities and an impaired ability to cause ALA in hamsters. Surprisingly, adhesion of these amebae to mammalian or bacterial cells was unchanged, suggesting that the light subunit plays a specific role in pathogenesis that does not involve adherence (82).

1.4.3 The Intermediate Subunit/150-kDa Surface Antigen

Several MAbs raised against an *E. histolytica* extract were shown to react against a 150-kDa surface protein (19), and one of them, named EH3015, significantly inhibited adherence of trophozoites to target cells, erythrophagocytosis and cytotoxicity *in vitro* (147). The immunoreacting molecule was found to be a lectin with Gal- and GalNAc-binding properties (148). Affinity purification using EH3015 results in co-purification of

the 150-kDa antigen together with the 260-kDa Gal-lectin, suggesting that these two molecules are physically associated. The 150-kDa antigen was therefore recently renamed "intermediate Gal-lectin subunit", or Igl. To date, two copies of the *igl* gene have been found in the ameba genome, and their encoded aa sequences showed 81% identity (149). The proteins are cysteine-rich, with amino- and carboxyl-terminal sequences typical of GPI-anchored membrane proteins.

1.4.4 Other Amebic Proteins Involved in Adherence

Incubation of amebae with Gal, GalNAc or anti-Gal-lectin antibodies does not completely inhibit adherence to target cells (118, 150), which suggests that the Gal-lectin is the major, albeit not the only amebic protein involved in adherence. A 220-kDa protein with lectin properties was isolated from *E. histolytica*, and was found to bind to MDCK cell monolayers; this adherence could be inhibited with micromolar concentrations of hyaluronic acid (HA), chitin and chitotriose, and with polyclonal antibodies raised against the molecule (151, 152). Another study showed that trophozoites bind to HA (found on the surface of epithelial cells and in the extracellular matrix) *via* an 80-kDa membrane protein that is recognized in immunoblots by two MAbs raised against human CD44, a HA receptor expressed by leukocytes (150). Amebae can also bind to the extracellular matrix *via* a 140-kDa protein that has fibronectin-binding activity. This molecule shows immunogenic crossreactivity with β1 integrins (153, 154), and a MAb raised against it inhibited adherence of trophozoites to the extracellular matrix (155). Finally, the 96-kDa EhADH2 protein, which is a bifunctional enzyme with alcohol dehydrogenase and

acetaldehyde dehydrogenase activities, was shown to bind to fibronectin and laminin (156).

1.5 Immune Responses Against *E. histolytica*

1.5.1 Systemic and Mucosal Humoral Immune Responses

The majority of *E. histolytica* infections results in antigenemia and stimulates a humoral antiamebic response (157). This response is very long lasting; ameba-specific serum antibodies have been shown to persist for up to 5 years after treatment (158, 159). These antibodies, which are mostly of the IgG (160), IgA, (161, 162), but also of the IgM (163, 164) classes, bind to a wide variety of amebic antigens (163, 165), including the serine-rich *E. histolytica* protein (SREHP) (166-168), but most importantly the Gal-lectin; the latter was the major trophozoite antigen recognized by sera from patients with ALA from different parts of the world (157, 169, 170). The CR of the heavy subunit (excluding the PR portion) is the most immunogenic region of the molecule: in one report, all samples tested recognized it, while there was 89 % seropositivity to the cysteine-poor region, and only 9% to the PR portion (171). The light subunit is very poorly immunogenic, demonstrated by the fact that antiserum raised against native Gal-lectin in mice did not react to it (123). As seropositivity to the SREHP and the Gal-lectin wanes off rapidly after ALA treatment, both proteins, in their recombinant forms, can be used in enzyme-linked immunosorbent assays (ELISAs) to effectively detect active infections in endemic areas (172). A specific mucosal antibody response is elicited by *E. histolytica* infections; anti-amebic sIgA was detected in the feces of patients with invasive amebiasis (162, 173-176), as well as in human milk (177) and saliva (178-181). This mucosal

response is long lasting (more than 12 months) (161). There seems to be no correlation between the levels of secretory and serum antiamebic sIgA in individuals who produce both (161).

Protection against ALA was achieved in several studies by passive immunization of laboratory animals with serum, purified polyclonal antibodies or MAbs against *E. histolytica* components (Table 1). However, immunization trials have demonstrated a lack of correlation between pre-challenge specific antibody titers and protection (182, 183). Similarly, the progression of ALA in human patients remains unaffected, despite the presence of high levels of antibodies early in the development of the disease (184), and reinfection is frequent in individuals with high anti-amebic antibody titers (185). Nevertheless, a correlation between the presence of anti-Gal-lectin intestinal sIgA and resistance to reinfection has recently been established (176).

Table 1. Passive immunization trials against experimental ALA

Antigen	Ab source	Animal model	% protection[a]	Ref.
SREHP	Rabbit	SCID mouse	100	(192)
LPG	Mouse MAb	SCID mouse	91.7[b]	(193)
150-kDa surface lectin	Mouse MAb	Hamster	75[b]	(194)
Gal-lectin - derived peptide	Rabbit	SCID mouse	67[b]	(195)
Gal-lectin CRD	Gerbil	Gerbil	0[b]	(143)
Whole amebae	Human patients	SCID mouse	29[b]	(196)

a: (number of immunized animals without ALA ÷ total number of immunized animals) - (number of control animals without ALA ÷ total number of control animals) x 100
b: the lesions in immunized animals with ALA were significantly smaller than those in the control animals

It is unclear how antibodies could confer protection against intestinal amebiasis and ALA. Adherence of amebae to neutrophils was enhanced when the former were pre-incubated in immune serum, but the amebicidal activity of the neutrophils was not increased (186). Anti-*E. histolytica* sIgA molecules in the gut (especially those against the Gal-lectin) could possibly prevent colonization by inhibiting adherence to the mucus

layer, or could aggregate trophozoites, making them easier to evacuate with the feces. It has been shown repeatedly that at 4°C serum antibodies that bind to membrane proteins on the surface of trophozoites can inhibit amebic adherence to target cells (170). However, at 37°C, amebae can aggregate, ingest, cap and shed immunoglobulins attached to them while remaining alive (187-189). The parasites can also rapidly and completely degrade human serum and secretory IgA (190) as well as IgG molecules (191) by means of CPs. Whether serum and/or mucosal antiamebic antibodies are important in resistance to reinfection remains to be determined.

1.5.2 Serum-Mediated Lysis and E. histolytica

Several reports state that *E. histolytica* trophozoites are susceptible to serum-mediated lysis. Serum lysozyme was able to kill amebae by an unknown mechanism (197), but its depletion did not completely eliminate ameba lysis. *E. histolytica* has been shown to activate complement *via* the alternative pathway (with the help of complement-fixing anti-amebic IgG) (198-201) and, more importantly, *via* the classical pathway (without the participation of specific antibodies) (200-203), due to the conversion of C3 to C3a by an amebic CP (202). However, trophozoites are resistant to complement-mediated lysis (201, 204), and this resistance correlates with *in vivo* virulence potential (205). The heavy subunit of the Gal-lectin shares sequence similarity and antigenic cross-reactivity with the human inhibitor of complement attack complex formation, CD59 (141, 206). Furthermore, it has been shown that purified, native Gal-lectin binds to the C8 and C9 components of the complement cascade and blocks assembly of the C5b-C9 complex, inhibiting formation of the cytolytic pore in the amebic membrane. A MAb raised against

the Gal-lectin neutralized this effect (141). While complement resistance seems to be low in amebae grown for a long time in usual culture medium, it can be regained by incubation with progressively higher concentrations of human serum (non heat-inactivated) (207, 208). This may perhaps correlate with an increase in Gal-lectin expression. Despite this complement resistance, it has been shown that *in vivo* inactivation of complement in *E. histolytica*-infected hamsters results in more severe amebic liver lesions, suggesting that complement may still play some role in controlling the development of the disease (209)

1.5.3 Cell-Mediated Immune Response

More and more evidence suggests that cellular immunity is of paramount importance in host defense and resistance to amebiasis; splenocytes, peripheral blood lymphocytes (PBLs) and peritoneal exudate cells (PECs) from ameba-immune hamsters all killed trophozoites *ex vivo* (210). While trophozoites readily kill unactivated leukocytes (110), they are susceptible to lysis by some of them when they are activated by certain cytokines. T cells and macrophages are the two main cell types effective against *E. histolytica* (211), although other cell types may also play a role, either due to their cytotoxic activities, or through their cytokine production.

1.5.3.1 T Lymphocytes

Early studies have shown that T cells play an important role in controlling *E. histolytica* infections. Thymectomy or *in vivo* depletion of T cells in animals infected with the parasite in the liver resulted in aggravation of pathology (212-214). In addition,

passive transfer of splenocytes from vaccinated or protected hamsters to naïve ones conferred protection against ALA, and depletion of T cells abrogated that effect (215). A T cell response develops early in extraintestinal *E. histolytica* infections; antigen-specific T cell proliferation and cytokine production are induced as early as 5 days post-infection (216)

It was found that amebic killing can be directly mediated by lymphocytes, *via* a major histocompatibility complex-nonrestricted mechanism (217). Two Lyt-23^+ mouse T cell clones raised against trophozoite antigen were able to efficiently kill amebae *in vitro* (218), and nonadherent splenocytes from Gal-lectin-immunized gerbils also had amebicidal activity (219). In humans, PBLs isolated from patients treated for ALA, and incubated with soluble amebic antigen (SAA) or native Gal-lectin, killed *E. histolytica* trophozoites (220, 221). In addition, phytohaemagglutinin-stimulated T cells from normal human patients showed amebicidal activity in a contact-dependent, Ab-independent manner. Depletion of $CD8^+$ T cells abrogated this activity (222), demonstrating that cytotoxic T lymphocytes were responsible. The overall *in vivo* contribution of these cells to anti-amebic immunity is, however, still unclear.

The main protective function of T cells in amebiasis is believed to be their cytokine production, which provides help for effector cell activation. It has been found that supernatants from T cells isolated from drug-cured ALA patients and restimulated with SAA or Gal-lectin were able to activate macrophages to kill trophozoites (220, 221). These cells, when restimulated with Gal-lectin, proliferated and produced the Th1 cytokines IL-2 and interferon (IFN)-γ (221), the latter being a potent macrophage activator. Similarly, gerbils immunized with purified Gal-lectin, which are protected

against a challenge infection in the liver (182), have splenocytes that secrete IL-2 and IFN-γ when restimulated *in vitro* with the same antigen (219). High IL-2 and TNF, and low IL-4 production by spleen cells was also associated with resolution of abscess in gerbils (216), further supporting the idea that development of a Th1 response is imperative for resistance. T cells may also provide B cell help for immunoglobulin production through their cytokine production. Nude mice (who are deprived of T cells) injected with live trophozoites fail to develop an IgG response against them, while their wild-type littermates do (223).

1.5.3.2 Macrophages

Macrophages are required for host defense against amebiasis. PECs from infected or immunized gerbils exerted a cytotoxic effect on trophozoites *in vivo* and *in vitro* (224). In addition, hamsters infected in the liver with virulent trophozoites and treated with BCG developed smaller abscesses, while those in infected animals treated with silica were bigger, with more metastases (225). Activated macrophages, which are the lymphoid cells with the highest amebicidal activity (224), kill amebae through a contact-dependent, antibody-independent and oxidative-dependent mechanism (110). *E. histolytica* trophozoites were found to be susceptible to oxidants, such as hydrogen peroxide (H_2O_2) (226), nitric oxide (NO) (227, 228), but not O_2^- (226). Another mechanism involving proteases may also be taking place (229).

Interferon-γ is central to resistance to amebiasis. A study using IFN-γ receptor knockout mice revealed the importance of this cytokine in innate immunity (230). While it is controversial whether IFN-γ has a direct, negative effect on trophozoite growth (229,

231), it is generally accepted that its main protective function lies in the activation of immune cells such as macrophages. Mouse and human macrophages incubated with IFN-γ killed trophozoites *in vitro* (229, 232-234). Lipopolysaccharide (LPS) acted synergistically with IFN-γ to enhance murine Kupffer cell amebicidal activity (234). However, the role IFN-γ plays in activating macrophages is not exclusive (232). TNF can also activate amebicidal activity in murine Kupffer cells (235), and IFN-γ, TNF, colony-stimulating factor-1, or combinations thereof, can all enhance the killing of trophozoites by murine macrophages (229). Transforming growth factor (TGF)-β_1 is able to prime mouse macrophages for enhanced amebicidal activity in response to IFN-γ with LPS or TNF (236).

While published studies suggest that oxidants, especially NO, are largely responsible for the tissue damage seen in ALA (237, 238), and that NO production is irrelevant to the direct elimination of trophozoites *in vivo* (238), another demonstrated, using iNOS knockout mice, that NO is required for the control of ALA (230). Macrophage NO, which is derived from L-arginine (227), has been shown several times to be cytotoxic against *E. histolytica* trophozoites *in vitro*. NO production is induced by the above mentioned cytokines; it is released from mouse macrophages stimulated with IFN-γ and LPS (228), and TNF turns on the *iNOS* gene in mouse macrophages for augmented NO production (239).

Extracts or purified antigens from *E. histolytica* have been found to directly activate macrophages. SAA injected into the peritoneal cavity of mice induced the recruitment of inflammatory macrophages that released H_2O_2 and O_2^- (240). In addition, pretreatment of mouse PECs with SAA primed them for enhanced H_2O_2 and O_2^-

production in response to phorbol myristate acetate (241). While SAA alone did not stimulate a respiratory burst (241), it was found to stimulate TNF mRNA production *via* a protein kinase C signal transduction pathway (242). Purified native Gal-lectin alone is able to activate macrophages. When added to mouse bone marrow derived macrophages, it stimulated TNF production by these cells (243) as well as amebicidal activity *via* NO when the cells were first primed by IFN-γ (244). The Gal-lectin also stimulated IL-12 p70 secretion by IFN-γ-primed human macrophages (245). This important finding may have applications in amebiasis vaccine development, as IL-12 is the principal cytokine involved in the differentiation of Th1 cells, and acts on T and natural killer (NK) cells to make them produce IFN-γ (246, 247), which further promotes Th1 differentiation.

1.5.3.3 Granulocytes

Although lysis of inflammatory cells by amebae at the site of infection results in extensive host cell death and tissue necrosis, granulocytes may play a significant role in host defense. Studies using neutropenic mice or neutrophil-depleted SCID mice revealed the importance of neutrophils, in particular in the early stages of ALA formation (248, 249). While nonactivated neutrophils are easily killed by virulent *E. histolytica* trophozoites by a contact-dependent mechanism (76), neutrophils activated by IFN-γ and/or TNF develop a marked amebicidal activity (250), killing trophozoites in a H_2O_2-dependent manner.

Unactivated eosinophils are easily killed by *E. histolytica* trophozoites (251), while those activated by f-Met-Leu-Phe kill virulent amebae with or without the help of opsonins (252), dying in the process. Gerbils rendered eosinophilic by the injection of

Toxocara canis antigen developed fewer and smaller ALAs after intrahepatic challenge (253), suggesting that these cells have a protective role.

1.5.3.4 Other Leukocytes

Despite their lack of T and B cells, SCID and nude mice do not get sustained infections with *E. histolytica*, and eventually clear out the parasites, even if large numbers of them are administered intrahepatically (223, 249). This indicates that cells other than T and B lymphocytes may also provide the cytokines necessary to activate effector cells. Natural killer cells may possibly secrete significant amounts of IFN-γ (254) in response to the proinflammatory cytokine IL-12 produced by dendritic cells and macrophages at the onset of infection.

1.6 Modulation of Host Immune Response and Survival Strategies

1.6.1 Suppression of Macrophages

More and more evidence suggests that *E. histolytica* is able to modulate the host's immune response in order to increase its own survival. Macrophages, the most important effector cells against trophozoites, appear to be the main target. Amebae secrete into their surroundings an oligopeptide that inhibits the *in vitro* locomotion of human monocytes as well as the respiratory burst in macrophages and neutrophils (255, 256). This monocyte locomotion inhibitory factor also inhibited delayed type hypersensitivity reaction in the skin of guinea pigs injected with 1-chloro-2-4 dinitrobenzene (257). Eicosanoids such as PGE_2 have downregulating effects on macrophage functions, and it was found that *E. histolytica* stimulates PGE_2 production in the liver during ALA formation in hamsters

(258). Treatment of the animals with the prostaglandin synthesis inhibitor indomethacin decreased the size of the abscesses. Trophozoites stimulated PGE_2 production in naïve gerbil PECs (259), and may also produce it themselves (260). While spleen and peritoneal macrophage functions are not significantly downregulated during the course of ALA development in gerbils, both effector and accessory cell functions are markedly affected in abscess-derived macrophages (261). These cells, when isolated during the acute phase of the infection (days 5 to 20), were found to be deficient in their capacity to develop a respiratory burst, to kill trophozoites *ex vivo*, to respond to IFN-γ, and to express IL-1-like activity (261). Trophozoites also suppressed IFN-γ-induced major histocompatibility complex (MHC) class II (Ia) molecule, I-Ab mRNA expression (262) and TNF production (263) in murine or gerbil macrophages by PGE_2-dependent mechanisms. Amebicidal activity is defective in ALA-derived macrophages, as *E. histolytica* modulates iNOS expression and NO production by these cells in a PGE_2-independent manner (264).

1.6.2 Suppression of Lymphocytes

Lymphocyte functions are also suppressed during invasive amebiasis. Amebic antigen affects the ability of murine splenocytes to proliferate in response to ConA (T cell mitogen) or LPS (B cell mitogen) (265). A yet unknown factor found in the serum of human patients with ALA (266) and gerbils 20 days after intrahepatic injection of live trophozoites (267) suppresses T cell proliferation in response SAA and ConA, respectively. In gerbils, this suppression was independent of NO and PGE_2, and involved a decreased IL-2 production by these cells, but not IL-2 responsiveness (267). T cell

suppression was also observed in mice immunized with a 220-kDa lectin (151), where the cells were unable to proliferate *ex vivo* in response to the purified antigen (268). These cells were nonetheless producing the Th2 cytokines IL-4 and IL-10, the latter being known to suppress T cell proliferation and IL-2 secretion (269).

As discussed above, *E. histolytica* evades complement-mediated lysis by means of the Gal-lectin, which confers complement resistance due to its homology to human CD59. Ameba CPs were found to degrade the complement-derived anaphylatoxins C3a and C5a (270); this allows trophozoites to limit the host inflammatory response raised against them. Finally, it was shown that the *E. histolytica* 29 kDa surface protein has H_2O_2-removing activity (271, 272), which may allow them to resist the H_2O_2 produced by activated leukocytes and help them survive inside the host.

1.7 Immunity to Amebiasis

There is no doubt that a high degree of protective immunity against amebiasis can be induced in laboratory animals. Gerbils with ALA that are cured with metronidazole become resistant to reinfection with *E. histolytica* trophozoites injected in the liver (216), and protection, either complete or partial, was induced in different animal species by injection with total amebic lysate, some of its fractions or purified antigens, or by vaccination (reviewed below). However, whether or not humans acquire protective immunity to invasive amebiasis after recovery from amebic colitis or ALA is still unclear. The few early epidemiological studies on amebiasis report conflicting results on this subject (185, 273, 274), due mainly to the fact that, at that time, *E. histolytica* and *E. dispar* were considered a single species. Reliable tools are now available to distinguish

the two morphologically similar amebae from serum (275) or stool samples (276-278). One of them was recently used to test a cohort of children in Bangladesh, and it was found that presence of stool anti-Gal-lectin sIgA was associated with resistance to reinfection with *E. histolytica* (176). Although more work needs to be done to fully understand protective immunity against amebiasis in humans, the latter study indicates that it does occur. Yet the duration of such acquired protection is still unknown.

1.8 Vaccination Against Amebiasis

Early studies on vaccination against *E. histolytica* infections consisted of injections of whole amebic antigen or its chromatographic fractions in complete Freund's adjuvant into laboratory animals, followed by intracecal or intrahepatic challenge with live trophozoites (279-281). In these studies, complete protection was achieved in animals vaccinated with high molecular weight amebic proteins. Only partial protection could be obtained with low molecular weight antigens or with whole amebic lysates. Hamsters injected intradermally with live axenic trophozoites were protected against intrahepatic challenge (282). Furthermore, intravenous injection of rabbits with *E. histolytica* soluble antigen mixed with trehalose-dimycolate (used as adjuvant) gave 100% survival after challenge (283).

1.8.1 Subunit Vaccines Against ALA

Although crude amebic extracts are somehow protective against ALA in animals, they still may contain undesirable molecules that could cause immunosuppression or stimulate the wrong cytokine profile (Th2), leading to a lack of protection or exacerbation

of disease in some individuals (268). Thus, more recent studies on vaccination against invasive amebiasis aimed at identifying single amebic proteins or portions thereof with protective properties. Four of them have been found so far and used in laboratory animals in vaccination trials against ALA. These trials are summarized in Table 2. Although some antigens and strategies seem more effective than others, it has to be kept in mind that factors such as the method of purification of the antigen, the antigen dose, the immunization regimen, the adjuvant, the route of immunization, the animal model as well as the dose and virulence of challenge amebae, are all variable parameters that can greatly influence the outcome. It is therefore difficult to compare protective efficacies between vaccination trials because of this lack of standardization.

The best studied *E. histolytica* protein with regards to vaccination is the Gal-lectin; its functions and properties make it a molecule of choice to be included in a subunit vaccine. Antibodies raised against it may be protective, as the Gal-lectin is the main molecule involved in colonization of the mucus layer and adherence to target cells, and is the molecule responsible for complement resistance. The Gal-lectin is very immunogenic and antigenically stable from one strain to another, two important characteristics for a vaccine candidate molecule; it was the main molecule immunoprecipitated by serum from patients with amebiasis, and MAbs raised against the Gal-lectin recognized the molecule in 16 *E. histolytica* strains isolated from different geographic areas (169). The molecule stimulates a humoral immune response and spleen cell proliferation in immunized animals, and therefore contains both B and T cell epitopes (182, 219, 284). While purified, native Gal-lectin conferred 67% protection against intrahepatic challenge with virulent trophozoites in the gerbil model of ALA

Table 2. List of subunit vaccine trials done against ALA

Antigen	Form[a]	Delivery/ Adjuvant	Route[b]	Model	Abs[c]	CMI[d]	% Eff.[e]	Ref.
Gal-lectin 260-kDa heterodimer	Native	Freund's	i.p.	Gerbil	+	+	67[f,g]	(182, 219)
Gal lectin aa 649 – 1201 (CR)	Recombinant, GST fusion	Freund's	i.p.	Gerbil	+	N/D	81	(285)
Gal-lectin aa 767 – 1138 (CR)	Recombinant, His-tagged	Titermax	i.p.	Gerbil	+	N/D	71[f]	(183)
Gal-lectin aa 482 – 1138 (CR)	Recombinant, His-tagged	Freund's	i.p.	Gerbil	N/D	N/D	45[f]	(286)
Gal-lectin aa 482 – 1138 (CR)	Recombinant, GST fusion	*Salmonella dublin*[h]	Orally	Gerbil	-	N/D	15.3[f]	(286)
Gal-lectin aa 1 – 442 (CP)	Recombinant	Freund's	i.p.	Gerbil	+	+	6.7[g]	(144)
Gal-lectin aa 442 – 630 (PR)	Recombinant	Freund's	i.p.	Gerbil	+	+	37.5[f]	(144)
Gal-lectin aa 805 – 945 (CR)	Recombinant	Freund's	i.p.	Gerbil	+	+	11.1	(144)
Gal-lectin aa 945 – 1059 (CR)	Recombinant	Freund's	i.p.	Gerbil	+	+	62.5[f]	(144)
Gal-lectin aa 895 - 998	Recombinant, His-tagged	Freund's	i.p.	Gerbil	+	N/D	0[f]	(143)
Gal-lectin aa 1005- 1029 (CR)	Synthetic, KLH fusion	Freund's	i.p.	Gerbil	+	N/D	33.3[f]	(195)
Gal-lectin aa 1005-1029 (CR)[i]	Recombinant, CtxB fusion	I- None II- Freund's	Orally i.p.	Gerbil	+ +	N/D N/D	0 – 30[f] 0 - 55	(195)
SREHP	Recombinant, MBP fusion	Freund's	i.p., i.d.	Gerbil	+	+	64 -100	(285, 287)
SREHP	Recombinant, MBP fusion	*S. typhimurium*[h]	Orally	Gerbil	+	N/D	77.8	(288)
SREHP	Recombinant	DNA vaccine	i.m.	Gerbil Mouse	+ +	+ +	60[f] 80[f]	(289)
29-kDa Ag	Recombinant, His-tagged	Titermax	i.p.	Gerbil	+	N/D	54	(290)
150/170kDa Ags	Native	I- Freund's II- Polyacrylamide	i.p.	Hamster	+ +	N/D	38[f] 67[f]	(291)

a: all recombinant proteins, except for the DNA vaccine-encoded SREHP, were produced in *E. coli*
b: i.p. = intraperitoneally, i.d. = intradermally, i.m. = intramuscularly
c: presence of antigen-specific antibodies in the serum of immunized animals. N/D = not determined
d: detection of antigen-specific T lymphocytes among splenocytes or positive delayed-type hypersensitivity reaction. N/D = not determined
e: (number of immunized animals without ALA ÷ total number of immunized animals) - (number of control animals without ALA ÷ total number of control animals) x 100
f: the lesions found in nonprotected immunized animals were significantly smaller than those in the control animals
g: the lesions found in nonprotected immunized animals were significantly larger than those in the control animals
h: Live, attenuated *Salmonella* spp expressing the antigen
i: antigen including this peptide, or portions thereof, fused to cholera toxin B subunit

(182), the CR region of the heavy subunit, which contains the CRD (143), was found to be the most protective portion of the molecule, the others being detrimental (144).

The SREHP is another immunogenic amebic protein that has been used in vaccination trials. It is a 25 kDa surface membrane protein that contains octapeptide and decapeptide tandem repeats that vary in number among *E. histolytica* strains (167, 292).

The function of the SREHP is still unknown, though it has been shown to be a potent chemoattractant for amebae (168). Vaccination with this protein induces a SREHP-specific humoral immune response (287, 288, 293-296), and antibodies against it inhibit adherence of trophozoites to target cells *in vitro* (296). High protection against intrahepatic challenge with live trophozoites has been achieved by vaccination with the SREHP, but the mechanism of this protection is still uncharacterized. A combination vaccine, including both recombinant Gal-lectin CR and recombinant SREHP conferred protection against experimental ALA in gerbils, but the efficacy was not superior to that obtained with either antigen alone (285).

The 29-kDa cysteine-rich protein (271), which is a molecule with H_2O_2-removal activity (272), and thus may be important for the survival of *E. histolytica* trophozoites within the host, has been reported to be recognized by sera from 80% of ALA patients living in Egypt and South Africa (290), and is therefore remarkably immunogenic. It conferred a vaccine efficacy of 54% in the gerbil model of amebiasis (290). Finally, the intermediate subunit of the Gal-lectin, or 150-kDa *E. histolytica* surface antigen (148, 149), has been considered as a vaccine candidate and, when administered intraperitoneally to hamsters, had a 38% or 67% protective efficacy against ALA, depending on the method of purification of the native antigen (291). Both preparations gave rise to serum antibodies that inhibited adherence of amebae to CHO cells.

A few other amebic antigens may be considered as potential vaccine candidates but have never been used in vaccination trials so far. These include the neutral CP (48) and the amebapore (102), which are two important virulence factors. The EhADH2 protein may also prove useful, as it is possibly involved in the adherence process, and is

an enzyme that plays a central role in the amebic fermentation pathway (156). Finally, the amebic lipophosphoglycan antigen (LPG), a surface molecule with high immunogenicity, may have vaccine potential (297); however, LPG does not contain a protein component, and the immunogenic epitopes are most likely carbohydrates, which makes large scale production of this molecule problematic.

1.8.2 Mucosal Immunization

As *E. histolytica* initially invades the host through a mucosal surface, it is believed that the ideal amebiasis vaccine should be able to stimulate a mucosal as well as a systemic immune response. It was shown that anti-amebic sIgA antibodies, purified from the saliva of patients with intestinal amebiasis, were able to inhibit adherence of trophozoites to MDCK cells *in vitro*, even though the amebae and the immunoglobulins were incubated together at 37°C for 1 hour (298). The recent association of the presence of anti-Gal-lectin stool sIgA with resistance to reinfection with *E. histolytica* stresses the importance of an amebiasis mucosal vaccine (176). Several studies have been published on this subject; however, because of a lack of a reliable animal model for intestinal amebiasis, these experiments were limited to the induction and characterization of ameba-specific sIgA in the gut, without possible *in vivo* challenge.

The cholera toxin B subunit has been the adjuvant of choice for induction of an amebic antigen-specific intestinal immune response. Whether administered to laboratory animals in combination with native 260-kDa Gal-lectin or with a portion of the cysteine-rich region of the heavy subunit (aa 767-1138), the adjuvant elicited an adherence-inhibitory Gal-lectin-specific sIgA response in the intestine (54, 299). Similarly, the

SREHP and its dodecapeptide repeat were expressed as a fusion protein with cholera toxin B subunit or the toxin's A_2 domain, and stimulated anti-SREHP stool sIgA when administered orally to mice (293, 295).

Delivery of the amebic antigen by a live, attenuated bacterial vector was another strategy used to elicit both systemic and mucosal immune responses. The SREHP fused to maltose-binding protein (MBP), or its dodecapeptide repeat fused to cholera toxin B subunit, were expressed in attenuated mutants of *Salmonella typhimurium* or *Vibrio cholerae*, respectively (288, 294). Both live vaccines stimulated a SREHP-specific mucosal immune response in orally immunized mice.

1.9 Animal Models for Amebiasis

Although some immunological studies on amebiasis can be done on cells and molecules isolated from humans with invasive amebiasis, others, including vaccine construction and trials, cannot be performed on humans until the late stages of development. Finding an adequate animal model for intestinal amebiasis and ALA has always been problematic, humans (and a few non-human primates) being the only hosts for *E. histolytica*. The poor availability of *E. histolytica* cysts has made things even more difficult since it has been impossible, to date, to generate cysts *in vitro*, and contaminated fecal matter from infected humans is their only source. The natural infection route thus has to be bypassed by delivering live trophozoites directly to the target organs.

A number of different animal species have been tentatively used as models for intestinal amebiasis, including kittens (300), dogs (301), rabbits (302), monkeys (303), guinea pigs (279), rats (304), and mice (211, 305). However, the results were variable and

in some cases irreproducible, as the amebae used were not axenic; they were cultured with an uncharacterized and variable human intestinal flora, and different isolates gave different results. Monoxenic amebae were used successfully to cause intestinal disease in gerbils (43, 306). Efforts to produce intestinal lesions in rats, guinea pigs, kittens and hamsters following inoculation of the large intestine with axenic *E. histolytica* cultures (including the virulent strain HM1:IMSS) have failed (307, 308). A method called "washed-closed cecal loop" has therefore been developed, where the cecum of the animals was closed using a silk thread, and its contents washed out before direct injection of axenic amebae into the cavity (308). This allowed the formation of cecal amebic abscesses in hamsters and guinea pigs (but not in kittens); however, only half of the inoculated animals developed pathology. While this method allows for the study of intestinal pathogenesis caused by *E. histolytica*, the low number of successful infections makes this model unfit for vaccination studies. Recently, a murine model of intestinal amebiasis has been established, which consists of infecting C3H/HeJ mice intracecally with axenic virulent trophozoites (309, 310). Although the animals develop intestinal disease, the problem of a low rate of infection (25%) remains. In brief, the lack of a reliable animal model for intestinal amebiasis is still a major obstacle, in particular for amebiasis mucosal vaccine research.

In hamsters and gerbils, progression of trophozoites from the ulcerated intestine to the liver may occur (306, 311) and cause pathologic changes in the liver, but does not always result in ALA; abscesses were present in 13 of 55 intracecally inoculated gerbils (306). Thus, to study ALA formation in these animals or protection thereof in vaccination studies, axenic virulent amebae (usually $5x10^5$) must be injected directly into the left liver

lobe (312, 313). Although both rodent species were shown to be susceptible to intrahepatic infection with live trophozoites (313, 314), *E. histolytica* is less virulent in gerbils and the liver lesions it causes in this animal resemble more those found in human ALA patients (315). The disadvantages of this method are that the animals may suffer complications due to surgery or anesthesia, and that intrahepatic challenge bypasses the mucosal immune system of the gut and its possibly important influence on the systemic immune response.

Because of a lack of immunological reagents specific for hamsters or gerbils, many researchers have been attempting to find a murine model of ALA. Early reports described the testing of several strains of mice for susceptibility to axenic *E. histolytica* intrahepatic infection, but all of the strains were found to be resistant; out of 209 mice of 9 different strains, only 8 developed liver lesions containing live amebae (314). Later, SCID mice, which are deficient in both T and B lymphocytes, were found to be susceptible to ALA when injected with high numbers (2.5×10^6) of virulent amebae into the liver (316); while all of the 7 SCID mice challenged developed amebic lesions with characteristics similar to those found in humans, only one of 7 immunocompetent congenic C.B-17 mice did. This animal model may be useful to study amebiasis with regards to passive immunization or lymphocyte reconstitution experiments, but it is obviously not of great use in vaccination studies, due to the absence of the most important cells involved in immune memory. One laboratory claims to have generated a mouse-adapted *E. histolytica* strain by performing multiple passages of HM1:IMSS trophozoites through mouse livers (94). These amebae, which have been only poorly documented, were able to infect immunocompromised as well as immunocompetent animals, and were

used to cause ALA in knockout mice of C57/BL6 background (94) and in C.B-17 mice (289).

E. histolytica generally behaves as a commensal in Old World monkeys, while it often is pathogenic in New World monkeys (303). Chimpanzees appear to be as susceptible to amebiasis as humans (317), yet because of high cost and low availability, they are not likely to ever be considered as an animal model. While one study described the induction of ALA in African Green Monkeys by intrahepatic injection of axenic *E. histolytica* trophozoites (318), a more recent one reported a failure to reproduce these results (296).

In summary, there are presently no perfect animal models for invasive amebiasis. None of the current models fulfills all the necessary criteria: a) the ability to be infected by the natural route; b) identical physiological and immunological reactions to infection as the ones seen in humans; c) being immunologically well characterized; and d) reasonable availability and cost.

1.10 The Mongolian Gerbil

The Mongolian gerbil (*Meriones unguiculatus*) is currently being used as an animal model in several medical fields such as neurology (319, 320), cancer research (321), and studies on infectious diseases. Its peculiar immune system makes it susceptible to a wide range of infections of viral (322), bacterial (323, 324), and mostly parasitic origins (42, 325-327). Ironically, very few immunological studies have been performed in gerbils, mainly due to a lack of gerbil-specific immunological reagents, such as recombinant cytokines and anti-cytokine monoclonal or polyclonal antibodies. In past

experiments, the assays mostly relied on basic phenomena such as lymphoproliferation (216, 328) and macrophage activation (325, 329), on reagents for other rodents (e.g. MAbs) that can react with gerbil molecules (216, 330, 331), or on cross-species bioassays when the gerbil cytokines studied were conserved enough to act on cells from other species, as it is the case for IL-2, IL-4 and TNF (216, 332). However, studies on the roles of gerbil molecules with high species specificity are currently impossible. While a few laboratories have recently published and/or submitted immunologically-relevant gerbil coding sequences (332, 333) and produced gerbil-specific reagents (334, 335), further efforts must be done to develop more reagents and make them commercially available.

The reason for the high susceptibility to numerous diseases observed in the gerbil is not known and, due to the above mentioned reasons, very little work has been conducted to understand it. It was recently reported that antibody production to T-independent antigens, such as dextran and phosphorylcholine, was impaired in gerbils (336), a phenomenon also observed in XID mice, which are deficient in Bruton's tyrosine kinase. Also similar to XID mice, the gerbils tested had no naturally occurring antibodies to single stranded DNA, LPS and phospholipids in their serum. It was thus suggested that gerbils may be deficient in B-1 ($CD5^+$) cells, a cell subset which is usually responsible for the production of such antibodies. Mongolian gerbil mutants with different coat colors were recently found to be differentially susceptible to *Brugia pahang*i infection (337). These mutants could be valuable tools to study the effect of genetic variation on immune responses to pathogens, and to better comprehend gerbil immunology.

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CHAPTER 2: DNA VACCINATION

DNA vaccination is the newest tool available in the field of vaccinology. Conventional vaccination relies on inactivated or attenuated whole organisms, and is quite successful at conferring protection against pathogens that can be controlled by an antibody response, such as bacteria and some viruses. However, it is of limited help against pathogens requiring a cell-mediated immune response for protection, such as many viruses, intracellular bacteria and intra- and extracellular parasites. DNA vaccines stimulate the development of both cell-mediated and humoral immune responses against an antigen, and their discovery has generated great enthusiasm among vaccine researchers.

2.1 Mechanisms of Immune Induction

The concept behind DNA vaccination is simple: a mammalian expression plasmid containing the DNA sequence coding for an antigen is administered to the subject (intradermally, intramuscularly or *via* a mucosal surface), and transfects host cells, which then produce the antigen (1). The resulting foreign protein elicits an immune response (2) that can subsequently protect against an infection with a pathogen that possesses the antigen (3). Induction of the immune response may occur by two major mechanisms, depending on the type of cell that produces the antigen. The first mechanism occurs when professional APCs (mainly dendritic cells) found in the skin, muscle or mucosal surface get transfected by the DNA vaccine (4, 5). The cells produce the antigen in their cytosol and, because the protein is intracellular, it gets degraded by proteasomes, and the peptide

products are displayed on the surface of the cells in the context of MHC class I molecules. These MHC class I/peptide complexes, in combination with co-stimulatory molecules expressed by the APCs such as CD80 (B7.1), CD86 (B7.2) and CD40, are then able to activate effector functions in cytotoxic T cells specific for the antigenic complexes (5-7). The second mechanism, which is believed to be the most significant (8), is known as "cross- priming" (9-11) and occurs when somatic cells, such as keratinocytes and myocytes, are transfected by the plasmid at the site of administration. These cells express the foreign antigen, which is "regurgitated" by them or released when the transfected cells die by apoptosis. The antigen is then taken up by APCs, which process it *via* the endocytic pathway and present its peptides in the context of MHC class II molecules to antigen-specific T helper cells. An alternative route of antigen processing also allows these exogenous antigens to be presented to cytotoxic T cells in the context of MHC class I (12-15). Transfected somatic cells could thus act as antigen reservoirs. Antigen presentation by somatic cells themselves may occur (3), but it is thought to be inefficient since the cells do not adequately express co-stimulatory molecules.

2.2 Administration of DNA Vaccines

Immunization with DNA vaccines may be accomplished through several methods and routes. Intramuscular or intradermal injection of DNA dissolved in a saline solution, and intradermal immunization using DNA-coated gold particles projected by means of a gene gun (16) are the two most popular strategies for the induction of a systemic immune response. However, the type of immune response induced and the quantity of DNA required for immunization differ for both methods. Saline-DNA administration with a

needle requires large amounts of plasmid (up to 200 μg per injection), as the frequency of *in vivo* transfection is very low and the great majority of the DNA is degraded by extracellular deoxyribonucleases (17). Irrespective of whether it is injected intramuscularly or intradermally, the vaccine, dissolve in saline solution and injected with a needle, generally induces a strong Th1 response (18). In contrast, gene gun-mediated DNA vaccination only requires nanogram quantities of plasmid DNA (19) since the frequency of transfection is very high, and it stimulates a Th2 response when done intradermally (18). The dose of DNA administered does not alone determine the type of immune response induced; it was observed that 1 μg of saline-DNA stimulated mostly IgG2a antigen-specific antibodies (indicative of a Th1 response), whereas mice receiving 1.5 μg of DNA administered by gene gun produced mostly IgG1 antibodies (Th2 response) (18). The reason for this differential induction of T helper cell phenotypes is still poorly understood. Other approaches of systemic or mucosal DNA vaccine delivery include liposomes (20, 21), cochleates (22), microparticle encapsulation (23) and attenuated bacteria such as *Shigella* (24), *Salmonella* (25), and *Listeria* (26).

2.3 Structure of DNA Vaccines

DNA vaccines consist of a foreign gene inserted in a mammalian expression vector. They all share some common features (Fig. 1): they must have a prokaryotic origin of replication and a bacterial antibiotic resistance gene to allow for construction, propagation and production of the vaccine in *Escherichia coli*. The ampicillin resistance gene is common in vaccines destined to be administered to laboratory animals; however, since it is prohibited for use in humans, the kanamycin resistance gene is often used

instead. The expression cassette of the vaccine is made of a strong promoter for expression in mammalian cells. This promoter, such as the human cytomegalovirus (hCMV) or the simian virus 40 (SV40) promoter, is usually derived from viral genes. The antigen coding sequence is inserted downstream from the promoter, and upsteam from a polyadenylation signal sequence for enhancement of mRNA transcript stability. Some DNA vaccine plasmids encode two antigens instead of a single one; in these cases, the plasmid may contain two separate expression cassettes. Alternatively, the genes can be inserted into a dicistronic expression vector, which encodes an mRNA that includes both coding sequences in tandem, separated by an internal ribosomal entry site (27).

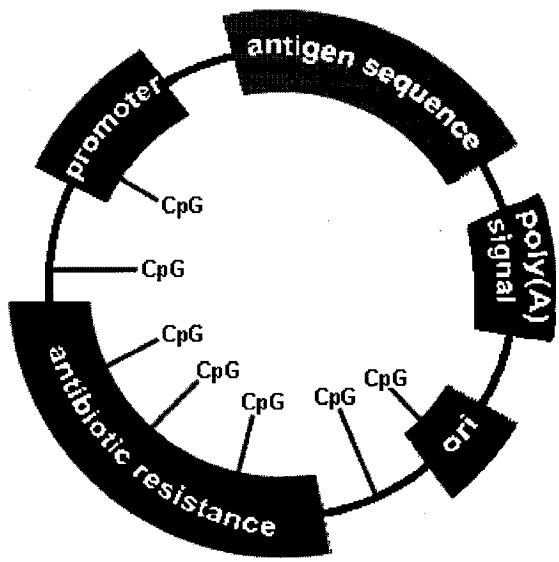

Figure 1. General structure and features of DNA vaccines.

2.4 CpG Motifs

In the early 1990s, it was observed that specific nucleotide sequences found in bacterial DNA had immunostimulatory activities (28, 29). Later, using a panel of synthetic oligonucleotides, these sequences were identified; they consist of an unmethylated cytosine-phosphate-guanine (CpG) dinucleotide flanked by two purine residues at the 5' end and two pyrimidines at the 3' end (30, 31). Methylation of CpG motifs abrogates the immunostimulatory effect (31). Mammalian DNA lacks this activity because, contrary to bacterial DNA, it contains a very low frequency of CpG dinucleotides (a phenomenon termed "CpG suppression"), and those that do occur tend to be methylated on the cytosine residue (32).

CpG motifs were found to directly stimulate polyclonal proliferation of B lymphocytes as well as their antibody production (31). They induce professional APCs to secrete proinflammatory cytokines, such as IL-6, IL-12, IL-18, TNF and IFN-α/β, and indirectly induce NK cell activation and IFN-γ production *via* APC-derived cytokines such as IL-12 and TNF (33-35). In addition, they activate dendritic cells and stimulate their growth and maturation (36). Finally, they directly or indirectly stimulate T lymphocytes, depending on their baseline activation state (37). Cell signalling in response to CpG motifs has recently been shown to be mediated by toll-like receptor-9 (38-40).

The cytokines induced by CpG motifs, notably IL-12, IL-18 and TNF, are potent stimulators of IFN-γ production by T and NK cells. Together, these cytokines stimulate the establishment of a Th1 response (41). Because of this immunostimulatory activity, synthetic oligonucleotides containing CpG motifs have been extensively used as experimental Th1 adjuvants for immunization with protein antigens against pathogens

(42) and cancer (43), or administered without antigen as therapy to treat asthma (44) and diseases requiring macrophage activation (45-47).

CpG motifs play an important role in the immunogenicity of DNA vaccines. The core of the plasmid normally contains several CpG motif sequences (Fig. 1) and, as DNA vaccines are prepared from *E. coli* cultures, those motifs are unmethylated and have immunostimulatory properties. Methylation of cytosine residues in CpG motifs present in a DNA vaccine abrogated its ability to stimulate a Th1 response (48), which demonstrates the adjuvant effect of the plasmid. Furthermore, adding supplemental CpG motifs into the plasmid backbone of a DNA vaccine enhanced its immunogenicity (48), but only up to a certain point; too many CpG motifs are detrimental, as they actually reduce the immunogenicity of the vaccine (49).

2.5 Genetic Adjuvants

DNA vaccination has been a very effective way to stimulate protective immunity in small laboratory animals. However, immunogenicity of DNA vaccines is relatively low in large animals (50) and in nonhuman primates (51). This could be due to the fact that CpG motifs are not as active on human immune cells as they are on murine cells (52). A promising method to further modulate the immune response to DNA immunization is the co-administration of the DNA vaccine with plasmids encoding biological adjuvants such as cytokines and co-stimulatory molecules (53). In 1993, it was found that injection of a cytokine gene into muscle tissue resulted in production of the bioactive cytokine by the transfected cells and enhancement of the immune response to a protein antigen (54). Since then, several research teams have repeated this novel method, applying it to DNA

vaccination (instead of protein injection) and using plasmids encoding a wide variety of immunologically active molecules (53). It was found, for instance, that co-administration of genes encoding IL-2, IL-12 or IL-18 enhanced the antigen-specific Th1 response, increased cytotoxic T lymphocyte (CTL) activity (55-57). Co-administration of IL-12 or IL-18, but not IL-12 genes also increased antibody production. Co-injection of plasmids encoding IL-4, IL-5 or IL-10 enhanced the Th2 response and IL-4 also decreased CTL activity (57). A GM-CSF construct either enhanced humoral immune response or not, depending on the time of injection relative to that of the antigen-encoding plasmid (58). Co-injection of the CD80 gene enhanced delayed type hypersensitivity (DTH) (59), while CD86 enhanced both CTLs and DTH (60). Administration of combinations of cytokine/co-stimulatory molecule constructs together with the DNA vaccine may also be performed (53), thus further increasing the range of modulatory effects on the specific immune response.

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SECTION II: MANUSCRIPTS I, II, III AND IV

MANUSCRIPT I

Construction and immunogenicity of a codon-optimized *Entamoeba histolytica* Gal-lectin-based DNA vaccine*

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ABSTRACT

Invasive amebiasis caused by *Entamoeba histolytica* is the third leading parasitic cause of mortality, and there are no vaccines available to help control the disease. The galactose-inhibitable adherence lectin (Gal-lectin) is the parasite's major molecule allowing it to adhere to colonic mucin for colonization and to target cells for tissue destruction. It is immunodominant and is regarded as the most promising candidate molecule to be included in a subunit vaccine against amebiasis. In this study, we are reporting the construction of a codon-optimized DNA vaccine encoding a portion of the Gal-lectin heavy subunit that includes the carbohydrate recognition domain, and its *in vivo* testing in mice. The vaccine stimulated a Th1-type Gal-lectin-specific cellular immune response as well as the development of serum antibodies that recognized a recombinant portion of the heavy subunit, and that inhibited the adherence of trophozoites to target cells *in vitro*.

INTRODUCTION

The protozoan parasite *Entamoeba histolytica* infects 50 million people worldwide and, despite the existence of effective antiamebic chemotherapy, causes up to 100,000 deaths from amebic colitis and/or amebic liver abscess (ALA) each year (1). There are currently no vaccines available to control or decrease the prevalence of the parasite. However, epidemiologic data suggest that a protective antiamebic immune response occurs in humans (2), and therefore that a vaccine against amebiasis could be possible. Such a vaccine could help eradicate amebiasis as humans are the only hosts for *E. histolytica*, and there are no known vectors to transmit the disease.

We have recently shown that gerbils with ALA that are cured with metronidazole become resistant to reinfection with *E. histolytica* trophozoites injected in the liver (3). In an earlier study, drug-cured ALA patients have been shown to develop an *in vitro* antiamebic cell-mediated immune response characterized by amebicidal cytotoxic T cells, T cell proliferation in response to amebic antigen, and lymphokine production of the Th1 type, especially interferon (IFN)-γ, which could effectively activate macrophages to kill trophozoites *in vitro* (4-6).

The Gal-lectin is the major surface molecule of *E. histolytica* that allows the parasite to adhere to colonic mucin and target cells (7), and is one of the parasite's most immunogenic molecules. It is the major antigen recognized by sera from patients with invasive amebiasis (8), and immunization of gerbils with the native protein or some of its portions confers protection against an intrahepatic challenge with live trophozoites (9-13). Moreover, monoclonal antibodies (MAbs) (14), antiserum (15) and secretory IgA (sIgA)

(16) against the Gal-lectin have been shown to inhibit adherence of trophozoites to colonic mucin and target cells. The Gal-lectin, or some of its selected portions, would thus be excellent candidate molecules to be included in a subunit vaccine.

DNA vaccination is an increasingly popular strategy to induce strong antibody and cell-mediated immune responses against a specific antigen, and has been used successfully to confer protection against a variety of pathogens. The unmethylated CpG motifs present in the backbone of the DNA vector elicit cytokine secretion by splenocytes, including interleukin (IL)-12 and tumor necrosis factor (TNF) (17), which promote the development of a Th1 immune response. Since a protective immune response against invasive amebiasis is also of the Th1 type (3), DNA vaccination could be an effective way to confer protection against this disease. The best animal model to study invasive amebiasis is the gerbil (18); however, there are limited immunological reagents to study cell-mediated and antibody responses in this animal. This is the first report of the construction and *in vivo* testing in mice of a codon-optimized Gal-lectin-based DNA vaccine.

MATERIALS AND METHODS

Vaccine construction

Making of pCIST vaccine vector

pCI-neo plasmid (Promega Corp., Madison, WI) was linearized by digestion with *Bam*HI, and the ends made blunt with T4 DNA polymerase (GIBCO, Burlington, Canada). Similarly, pDisplay (Invitrogen, Carlsbad, CA) was linearized with *Afl*III and

made blunt at both ends. Both plasmids were then digested with *Eco*RI, and the 3184 bp fragment of pCI-neo, which contains the Amp^r gene, the bacterial origin of replication, the hCMV enhancer/promoter element and the chimeric intron, was ligated to the 666 bp fragment of pDisplay, which contains the Ig κ- chain secretion peptide, the hemagglutinin A epitope, the multi cloning site, the *myc* epitope, the PDGFR transmembrane domain and the BGH poly(A) addition site sequences. The resulting 3851 bp plasmid was named pCIST and was used as vector for our DNA vaccine.

Codon optimization of the GL6 coding sequence

The method used to synthesize the optimized GL6 DNA sequence was derived from a previously published protocol (19). Since the DNA vaccine was initially destined to be tested in gerbils (*Meriones unguiculatus*), a table of synonymous codon usage in known gerbil cDNA sequences was made (Table 1) using the Codon Usage Database website (www.kazusa.or.jp/codon/), and the DNA encoding amino acids (aa) 894 – 1081 of the Hgl1 protein was completely rewritten using the preferred codons only (Fig. 1). The synthetic sequence was then subdivided in 4 segments by inserting unique restriction sites in it, without altering the amino acid sequence, by modifying a few of the codons, changing them for the second more frequent triplet. These restriction sites (*Cla*I, *Hind*III and *Sal*I) were carefully chosen so that they are not found anywhere else in the synthetic sequence, and that they are present in the multiple cloning site of the cloning vector pBluescript II SK+ (Stratagene, La Jolla, CA). Each of the four segments, which are of about the same length, was then synthesized separately. For each segment, a pair of oligonucleotides (Table 2) that contain 16-bp complementary sequences at their 3' end

Table 1. Codon usage in gerbil cDNA sequences

aa	Codon[a]	%[b]	aa	Codon[a]	%[b]	aa	Codon[a]	%[b]	aa	Codon[a]	%[b]
A	GCA	22.4	C	**TGC**	60.1	E	GAA	45.9	G	GGA	28.1
	GCC	33.8		TGT	39.9		**GAG**	54.1		**GGC**	33.0
	GCG	8.3	D	**GAC**	55.7	F	**TTC**	60.1		GGG	21.9
	GCT	35.5		GAT	44.3		TTT	39.9		GGT	17.0
H	**CAC**	54.5	K	AAA	38.9	L	CTA	7.4	P	CCA	28.2
	CAT	45.5		**AAG**	61.1		CTC	22.7		**CCC**	34.8
I	ATA	18.6	M	**ATG**	100		**CTG**	41.5		CCG	11.2
	ATC	51.7	N	**AAC**	62.0		CTT	11.8		CCT	25.8
	ATT	29.7		AAT	38.0		TTA	4.6	Q	CAA	30.9
R	**AGA**	31.0	S	**AGC**	30.5		TTG	12.0		**CAG**	69.1
	AGG	22.4		AGT	13.5	T	ACA	23.8	V	GTA	9.0
	CGA	9.4		TCA	14.3		**ACC**	38.5		GTC	28.2
	CGC	14.3		TCC	24.2		ACG	11.2		**GTG**	46.8
	CGG	18.0		TCG	3.6		ACT	26.5		GTT	16.0
	CGT	4.9		TCT	13.8	W	**TGG**	100	Y	**TAC**	61.9
										TAT	38.1

[a] Synonymous codons for the corresponding amino acid. The preferred codon for each amino acid is shown in bold characters.
[b] Percentage frequencies of the synonymous codons for each corresponding amino acid.

```
O  GAG GCC TAC TGC ACC TAC GAG ATC ACC ACC AGA GAG TGC AAG ACC TGC AGC CTG ATC
W  GAA GCA TAT TGT ACA TAC GAA ATA ACA ACA AGA GAA TGT AAA ACA TGT TCA TTA ATT
     E   A   Y   C   T   Y   E   I   T   T   R   E   C   K   T   C   S   L   I

O  GAG ACC AGA GAG AAG GTG CAG GAG GTG GAC CTG TGC GCT GAG GAG ACC AAG AAC GGC
W  GAA ACT AGA GAA AAA GTC CAA GAA GTT GAT TTG TGT GCA GAA GAA ACT AAG AAT GGA
     E   T   R   E   K   V   Q   E   V   D   L   C   A   E   E   T   K   N   G
                                                    ClaI
O  GGC GTG CCC TTC AAG TGC AAG AAC AAC AAC TGC ATC ATC GAT CCC AAC TTC GAC TGC
W  GGA GTT CCA TTC AAA TGT AAG AAT AAC AAT TGC ATT ATT GAT CCT AAC TTT GAT TGT
     G   V   P   F   K   C   K   N   N   N   C   I   I   D   P   N   F   D   C

O  CAG CCC ATC GAG TGC AAG ATC CAG GAG ATC GTG ATC ACC GAG AAG GAC GGC ATC AAG
W  CAA CCT ATT GAA TGT AAG ATT CAA GAG ATT GTT ATT ACA GAA AAA GAT GGA ATA AAA
     Q   P   I   E   C   K   I   Q   E   I   V   I   T   E   K   D   G   I   K

O  ACC ACC ACC TGC AAG AAC ACC ACC AAG GCT ACC TGC GAC ACC AAC AAC AAG AGA ATC
W  ACA ACA ACA TGT AAA AAT ACT ACA AAA GCA ACA TGT GAC ACT AAC AAT AAG AGA ATA
     T   T   T   C   K   N   T   T   K   A   T   C   D   T   N   N   K   R   I
                 HindIII
O  GAG GAC GCT AGA AAA GCT TTC ATC GAG GGC AAG GAG GGC ATC GAG CAG GTG GAG TGC
W  GAA GAT GCA CGT AAA GCA TTC ATT GAA GGA AAA GAA GGA ATT GAG CAA GTA GAA TGT
     E   D   A   R   K   A   F   I   E   G   K   E   G   I   E   Q   V   E   C

O  GCT AGC ACC GTG TGC CAG AAC GAC AAC AGC TGC CCC ATC ATC ACC GAC GTG GAG AAG
W  GCA AGT ACT GTT TGT CAA AAT GAT AAT AGT TGT CCA ATT ATT ACT GAT GTA GAA AAA
     A   S   T   V   C   Q   N   D   N   S   C   P   I   I   T   D   V   E   K
                             SalI
O  TGC AAC CAG AAC ACC GAG GTC GAC TAC GGC TGC AAG GCT ATG ACC GGC GAG TGC GAC
W  TGT AAT CAA AAC ACA GAA GTA GAT TAT GGA TGT AAA GCA ATG ACA GGA GAA TGT GAT
     C   N   Q   N   T   E   V   D   Y   G   C   K   A   M   T   G   E   C   D

O  GGC ACC ACC TAC CTG TGC AAG TTC GTG CAG CTG ACC GAC GAC CCC AGC CTG GAC AGC
W  GGT ACT ACA TAT CTT TGT AAA TTT GTA CAA CTT ACT GAT GAT CCA TCA TTA GAT AGT
     G   T   T   Y   L   C   K   F   V   Q   L   T   D   D   P   S   L   D   S

O  GAG CAC TTC AGA ACC AAG AGC GGC GTG GAG CTG AAC AAC GCT TGC CTG AAG
W  GAA CAT TTT AGA ACT AAA TCA GGA GTT GAA CTT AAC AAT GCA TGT TTG AAA
     E   H   F   R   T   K   S   G   V   E   L   N   N   A   C   L   K
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Figure 1. Amino acid sequence (residues 894 – 1081) of the portion of the Gal-lectin heavy subunit (Hgl1) included in protein GL6, and comparison of the wild-type (W) and optimized (O) DNA sequences coding for them. The nucleotides that have been changed for optimization are shown in italicized characters. The restriction enzyme sites that have been inserted to facilitate the synthesis of the optimized DNA are underlined and identified.

was annealed (1 μg each oligonucleotide) in 1 x Sequenase buffer (Amersham Pharmacia Biotech, Baie d'Urfé, Canada) by heating the reaction at 70°C for 5 minutes, and cooling it down to an annealing temperature calculated according to the base composition of the pair's complementary sequence. The reactions were then extended by adding each dNTP to a final concentration of 2.5mM and 10 U Sequenase v2.0 (Amersham), and incubating for 30 min at 37°C. The enzyme was inactivated by increasing the temperature to 70°C for 10 minutes, the synthesized DNA segments digested with the appropriate restriction enzymes (Table 2) and inserted in pBluescript II SK+ which was pre-treated with the same enzymes. Each of the four segments was then sequenced to ensure no mistakes were introduced during synthesis. Each segment was then digested out of the plasmid with the restriction enzymes used to put it in, and purified by agarose gel electrophoresis. Finally, the four segments were pooled together, ligated using T4 DNA ligase (GIBCO), and the ligation products used as template for PCR using the primers "oGL6 sense": 5'-CTA *GGG CCC* TGG AGG CCT ACT GCA CCT ACG-3' and "oGL6 antisense": 5'-*GGG* GCT CAC TTC AGG CAA GCG TTG TTC-3', and *Pwo* DNA polymerase (Boehringer Mannheim, Laval, Canada), according to the manufacturer's instructions. The 595-bp product was digested with *Apa*I, and inserted into the vaccine vector pCIST pre-treated with *Apa*I and *Sma*I, to yield the DNA vaccine pCISToGL6. Since a stop codon was inserted immediately at the end of the oGL6 sequence, the resulting protein does not include the transmembrane domain encoded by the vector. Both pCIST and pCISToGL6 were propagated in *Escherichia coli* DH5α, and purified by CsCl gradient centrifugation (20) from overnight cultures. Final DNA pellets were dissolved in phosphate-buffered saline, pH 7.4, at a concentration of 2 μg/ml.

Table 2. List of the oligonucleotides used to synthesize the optimized DNA sequence encoding protein GL6

Oligo[a]	Sequence[b]	Enzyme
A	5'-CT *GGA TCC* GAG GCC TAC TGC ACC TAC GAG ATC ACC ACC AGA GAG TGC AAG ACC TGC AGC CTG ATC GAG ACC AGA GAG AAG GTG CAG GAG GT-3'	*BamHI*
B	5'-CT *ATC GAT* GAT GCA GTT GTT GTT CTT GCA CTT GAA GGG CAC GCC GCC GTT CTT GGT CTC CTC AGC GCA CAG GTC CAC CTC CTG CAC CTT CT-3'	*ClaI*
C	5'-GCT *ATC GAT* CCC AAC TTC GAC TGC CAG CCC ATC GAG TGC AAG ATC CAG GAG ATC GTG ATC ACC GAG AAG GAC GGC ATC AAG ACC ACC A-3'	*ClaI*
D	5'-GCT *AAG CTT* TTC TAG CGT CCT CGA TTC TCT TGT TGT TGG TGT CGC AGG TAG CCT TGG TGG TGT TCT TGC AGG TGG TGG TCT TGA TGC C-3'	*HindIII*
E	5'-GCT *AAG CTT* TCA TCG AGG GCA AGG AGG GCA TCG AGC AGG TGG AGT GCG CTA GCA CCG TGT GCC AGA ACG ACA A-3'	*HindIII*
F	5'-GCT *GTC GAC* CTC GGT GTT CTG GTT GCA CTT CTC CAC GTC GGT GAT GAT GGG GCA GCT GTT GTC GTT CTG GCA CA-3'	*SalI*
G	5'-GCT *GTC GAC* TAC GGC TGC AAG GCT ATG ACC GGC GAG TGC GAC GGC ACC ACC TAC CTG TGC AAG TTC GTG CAG CTG ACC GAC GAC CCC-3'	*SalI*
H	5'-CAT *GCG GCC GCT* ACT TCA GGC AAG CGT TGT TCA GCT CCA CGC CGC TCT TGG TTC TGA AGT GCT CGC TGT CCA GGC TGG GGT CGT CGG TCA GC-3'	*NotI*

[a] Oligos A and B, C and D, E and F, and G and H are complementary to each other.
[b] The 3' complementary sequences are underlined, and the restriction enzyme recognition sequences are italicized.

Expression of GL6 in COS-7 cells transfected with pCISToGL6

COS-7 cells (10^6) were transfected with pCISToGL6 or pCIST (5 μg) using LipofectAmine (GIBCO), and subsequently incubated for 72 hours in 5 ml DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 24 mM HEPES, at 37°C in a humidified 5% CO_2 atmosphere. The cells were then harvested and dissolved in 50 μl reducing SDS-PAGE buffer, while the supernatants were concentrated 20:1 by lyophilization and a 50-μl aliquot mixed with an equal volume of 2X SDS-PAGE buffer. Twenty microliters of each cell lysate and supernatant were separated in a 10% SDS-polyacrylamide gel. Recombinant protein GL5, a poly-histidine-tagged portion of the heavy subunit of the Gal-lectin (aa 754-1137), was also run as control for immunoreactivity. The proteins were transferred onto a nitrocellulose membrane, and the blot probed with 3F4 hybridoma cell culture supernatants, which recognize an epitope within aa 895-998 of the Gal-lectin's heavy subunit (21). The blot was then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse Ig antibody (Amersham), and developed by enhanced chemiluminescence (ECL; Amersham).

Vaccination

Female, 42 days old Balb/c mice (Charles River, St-Constant, Canada), 3 per group, were injected intradermally, on the back, with 100 μg of plasmid, in a volume of 50 μl. Identical booster injections were performed after 14 and 28 days. Ten days after the last injection, the mice were sacrificed, and their spleens and sera were collected.

Lymphoproliferation assay

Single-cell suspensions were prepared from the vaccinated animals' spleens (pooled within the same group), by passing the organs through a wire mesh, in RPMI 1640 (GIBCO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 24 mM HEPES, 50 μM β-mercaptoethanol, and containing 1% fetal bovine serum (FBS). The red blood cells were lysed by osmotic shock using Gey's salts (22), and the remaining cells were resuspended at a density of $5x10^6$ /ml in complete RPMI (as above, but containing 10% FBS). The cell suspensions (100 μl/well) were distributed in a flat-bottomed 96-well culture plate (Falcon; Becton Dickinson, Lincoln Park, NJ), and an equal volume of stimulus was added to the wells, in triplicates. The stimuli used for this experiment were: complete RPMI medium (no stimulus); 10 μg/ml immunoaffinity-purified native Gal-lectin (kindly provided by Dr. Barbara Mann, University of Virginia); 50 μg/ml soluble amebic antigen (SAA), prepared as previously described (3); or 5 μg/ml ConA (Pharmacia, Uppsala, Sweden). The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 48 hours, after which 1 μCi [^{3}H]thymidine (ICN, Montreal, Canada) was added to each well, in 25 μl complete RPMI. The cultures were incubated for another 18 hours and harvested onto glass fiber filters, using an automated cell harvester (Skatron, Lier, Norway). The radioactivity incorporated in the cells was finally measured by scintillation counting (LKB Wallac; Pharmacia).

Immunoblot

The sera from the vaccinated mice were tested by immunoblot for presence of anti-Gal-lectin antibodies. Electrophoresis was performed on recombinant protein GL5 (1

μg/lane) in a 10% SDS-polyacrylamide gel and the antigen was transferred onto a nitrocellulose membrane. The lanes were probed with a 1:250 dilution of pooled sera from the mice vaccinated with pCISToGL6, those injected with pCIST or, as positive control, with undiluted 3F4 hybridoma culture supernatant. The blots were then incubated with HRP-conjugated anti-mouse Ig antibody, and developed by ECL.

Antibody isotype enzyme-linked immunosorbent assay (ELISA)

To measure Gal-lectin-specific IgG1, IgG2a, IgG3 and IgE antibodies in the serum from the vaccinated mice, wells from Immulon 2 "U" bottom plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 1 μg recombinant protein GL5 in 100 μl 50mM carbonate buffer (pH 9.6). The plates were washed with PBS-0.1% Tween20, blocked overnight at 4°C with 200 μl PBS-1% BSA (Sigma Chemical Co., St-Louis, MO), and washed again. Serum samples were serial diluted 1:2 in PBS-1% BSA, distributed in triplicates in the wells, and incubated at 37°C for 1 hour. The plates were washed again and 100 μl of a biotin-labeled anti-mouse IgG1, IgG2a, IgG3 or IgE antibody (Pharmingen, San Diego, CA; 1:1000 dilution) was added to each well for 1 hour at 37°C. After washing, 100 μl streptavidin-peroxidase (Pharmingen; 1:2000 dilution) were added to the wells for 20 minutes, the wells were washed and the assay developed using 100 μl *o*-phenylenediamine dihydrochloride substrate (Sigma). The reactions were stopped with 50 μl 2N H_2SO_4 and absorbance was read at 450/630 nm in a Dynatech MR5000 microplate reader (Dynatech).

Adherence assay

The effect of sera from the vaccinated animals on amebic adherence to target cells was evaluated by adherence assay, as described previously (23). Briefly, log phase *E. histolytica* trophozoites (strain HM1-IMSS), grown in TYI-S-33 medium (24), were harvested, washed in M199 medium (GIBCO) and resuspended in M199 at a concentration of 10^5/ml. One milliliter aliquots were incubated at 4°C for 1 hour with a 1:100 dilution of pooled sera from: the mice injected with pCISToGL6; those injected with pCIST; immune serum from ALA patients (provided by J. Keystone, University of Toronto); or normal human subjects. Meanwhile, Chinese hamster ovary (CHO) cells, grown in Ham's F12 medium (GIBCO) supplemented with 10% FBS, were trypsinized and resuspended in M199 at a density of $2x10^5$/ml. Amebae (10^4) were then mixed with 1 ml CHO cell suspension, the cells were centrifuged (500 x *g* for 5 min), and after a 2-hour incubation at 4°C, 850 µl of the supernatant were removed, and the pellet lightly resuspended in the remaining medium. Amebic adherence to CHO cells was then assessed by determining by light microscopy the number of amebae with three or more CHO cells attached to them (positive rosette) out of 100 amebae selected at random.

Statistical analysis

Student's *t*-test was used to identify differences between experimental groups and their controls. $P < 0.05$ was considered significant. All values are expressed as mean ± SE.

RESULTS

Construction of the pCISToGL6 vaccine and expression of the antigen in COS-7 cells

DNA encoding aa 894 – 1081 of the Hgl1 protein (protein GL6) was synthesized *de novo* using, for each amino acid residue encoded, the most frequent synonymous codon found in gerbil coding sequences for that amino acid (Fig. 1). This resulted in a coding sequence with a G:C content of 58%, compared to 30% for the wild-type sequence. The codon-optimized coding sequence was then inserted in a vaccine vector, pCIST, containing elements allowing for high protein expression in mammalian cells. COS-7 cells were then transfected with this vaccine construct, pCISToGL6, and produced a protein with an apparent molecular weight of 40 kDa that immunoreacted with MAb 3F4 in an immunoblot (Fig.2). This protein was partially soluble, as it was detected in the supernatant from the transfected cells, but mostly in the cell pellet; its poor solubility is probably due to the high number of cysteine residues in the protein (18), potentially

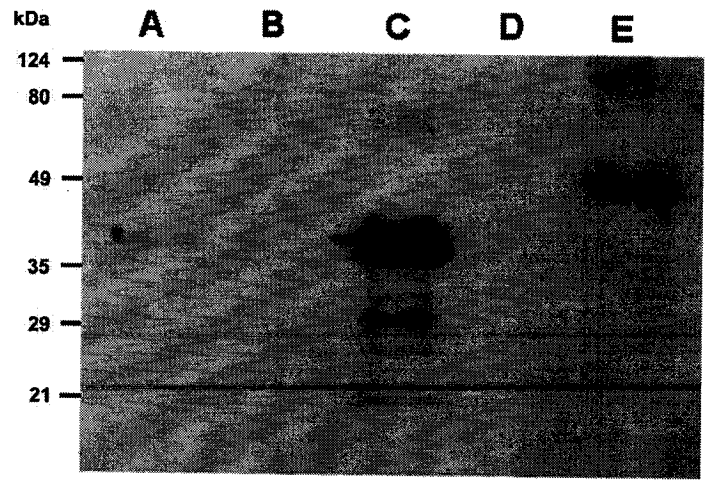

Figure 2. *In vitro* expression of protein GL6 by transfected mammalian cells. COS-7 cells were transfected with the DNA vaccine or the control vector, and analyzed by immunoblot for expression of the Gal-lectin-derived antigen. Lanes A and B: concentrated supernatants of cells transfected with pCISToGL6 and pCIST, respectively. Lanes C and D: lysates from cells transfected with pCISToGL6 and pCIST. Lane E: recombinant protein GL5. The blot was probed with undiluted 3F4 hybridoma cell culture supernatant.

forming intermolecular disulfide bonds. Cells transfected with the pCIST plasmid did not produce such a protein.

pCISToGL6 induces a Gal-lectin-specific cellular immune response in mice

A simple way to determine whether a vaccine stimulates an antigen-specific immune response is to measure the proliferation of peripheral blood mononuclear cells or spleen cells in response to the pure antigen or to pathogen extracts containing the antigen. As shown in Figure 3, pooled spleen cells from the mice vaccinated with pCISToGL6 proliferated markedly in response to Gal-lectin-containing SAA (15, 25) (11,349 ± 495

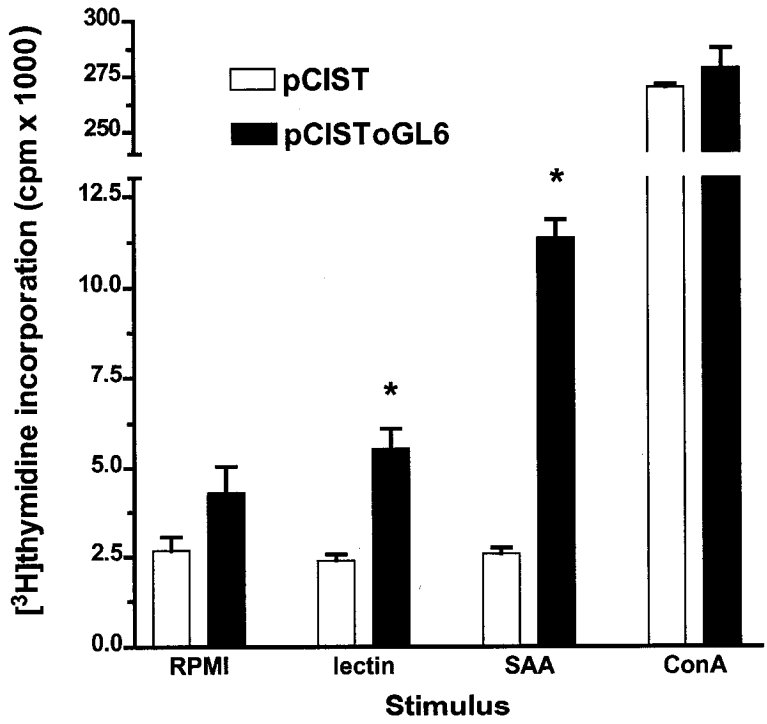

Figure 3. Gal-lectin-specific lymphoproliferation of splenocytes from the mice vaccinated with pCISToGL6. Spleen cells from the vaccinated and control animals were incubated *in vitro* with native Gal-lectin, SAA, ConA or medium alone, and their proliferation measured by [^{3}H]thymidine incorporation. An asterisk indicates a significant difference between the two groups for a given stimulus ($p < 0.05$).

cpm) and, to a lower extent, to purified Gal-lectin (5,532 ± 567 cpm), compared to splenocytes from the control mice (2,581 ± 156 cpm and 2,390 ± 167 cpm for SAA and Gal-lectin, respectively). The higher proliferative response to SAA may be due to a higher final concentration of Gal-lectin in the SAA culture wells compared to the purified Gal-lectin wells. Lymphoproliferation in response to the T cell mitogen ConA was comparable for both groups (278,429 ± 9,053 for pCISToGL6 and 269,960 ± 1,180 for pCIST), showing that they were of equal viability.

Induction of Gal-lectin-specific, Th1-type serum antibodies by pCISToGL6

To determine whether pCISToGL6 stimulated the production of anti-Gal-lectin antibodies in the vaccinated animals, we probed nitrocellulose membranes containing protein GL5 (1 μg/lane), with a 1:250 dilution of their sera (pooled within each group), or with 3F4 hybridoma culture supernatant as positive control. Sera from pCISToGL6-vaccinated mice recognized GL5, whereas those from the mice injected with pCIST did not (Fig. 4). As expected, 3F4 supernatants strongly detected the recombinant amebic antigen. Antibody isotype-specific ELISAs performed on the sera revealed that pCISToGL6 induced a Th1-type immune response against the Gal-lectin, as only antigen-specific IgG2a antibodies were detected (Fig. 5). Their level was however quite low; they were completely titrated out at a 1:512 dilution.

Adherence-inhibitory effect of sera from mice vaccinated with pCISToGL6

Monoclonal and polyclonal antibodies against the lectin have been shown to have adherence inhibitory effects on *E. histolytica* trophozoites to target cells (14, 15). To

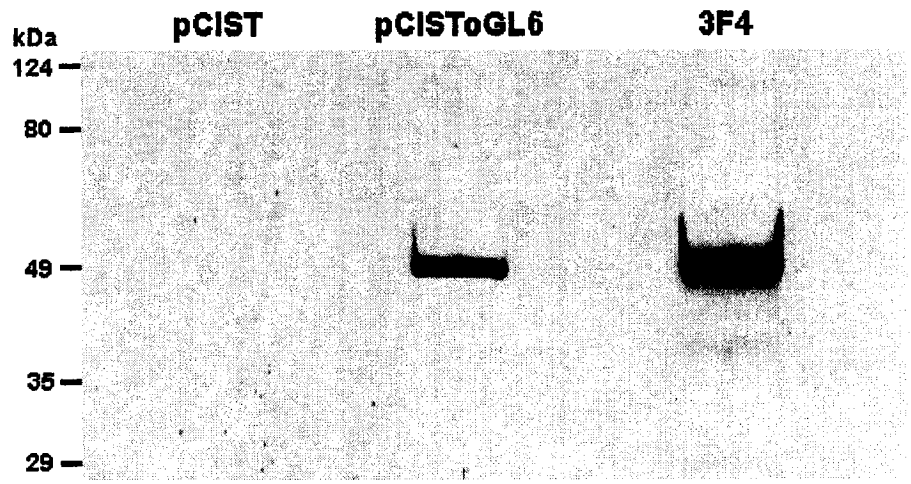

Figure 4. Pooled sera from the mice vaccinated with pCISToGL6 contain anti-Gal-lectin antibodies. Recombinant protein GL5 on nitrocellulose membranes was probed with sera from the control or vaccinated mice (1:250 dilution), or with undiluted 3F4 hybridoma supernatant.

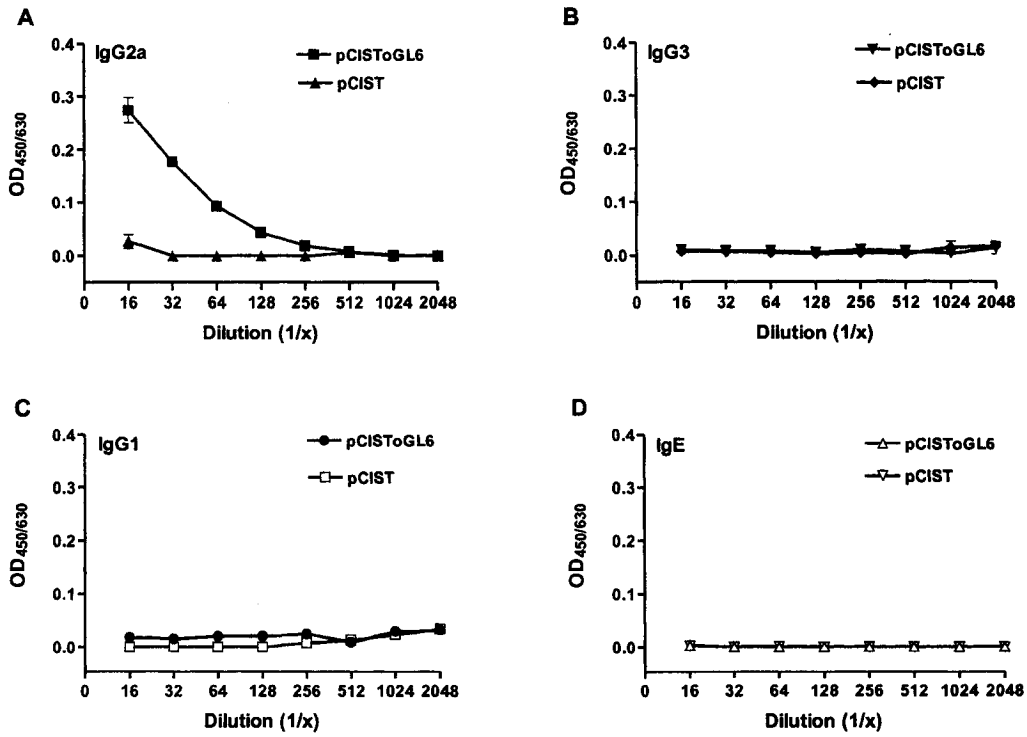

Figure 5. Serum anti-Gal-lectin antibodies of different isotypes induced by vaccination with pCISToGL6. Levels of antigen-specific IgG2a (A), IgG3 (B), IgG1 (C) and IgE (D) antibodies in the sera from the animals injected with pCISToGL6 or pCIST were estimated by ELISA, using recombinant protein GL5 to coat the wells.

determine whether the anti-Gal-lectin antibodies in the sera of the vaccinated mice show similar effects, we tested them in an *in vitro* model of the disease. As shown in Figure 6, while sera from control mice (and normal human serum) had no effect on adherence of trophozoites to CHO cells, sera from the animals vaccinated with pCISToGL6 inhibited rosette formation by 29%. By comparison, sera obtained from patients with ALA inhibited trophozoites adherence by 94%. All sera were diluted 1:100 for this assay.

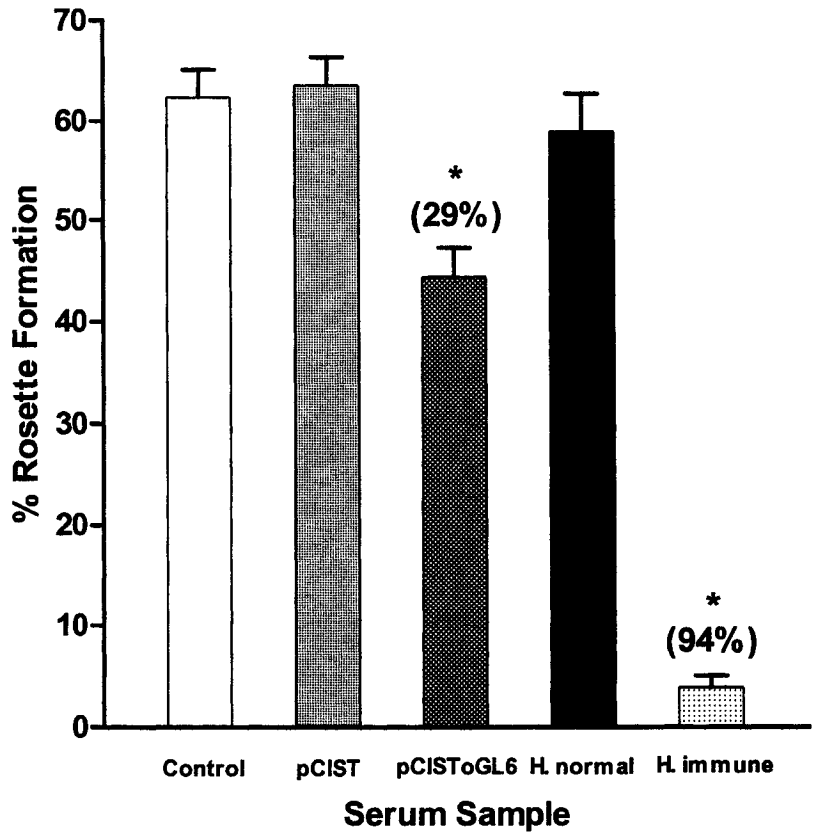

Figure 6. Amebic adherence inhibitory activity of sera from the mice vaccinated with pCISToGL6. CHO cell adherence assay was performed as described in Materials and Methods, using trophozoites pre-incubated with serum (1:100 dilution) from mice (injected with pCIST or pCISToGL6) or humans (normal or with ALA). Control amebae were pre-incubated with medium alone. Percentage of rosette formation was then determined for each condition. Asterisks indicate a significant difference between experimental groups and control group ($p < 0.5$), and percent decrease in amebic adherence compared to control is indicated between brackets.

DISCUSSION

Expression of foreign genes in mammalian cells can be problematic, especially when the codon usage biases between the gene of interest and normal mammalian genes differ substantially. A few reports have demonstrated that establishing a suitable codon bias for mammalian expression can dramatically increase protein production (26, 27). In the context of DNA vaccination, this can in turn enhance the immune response induced by the vaccine (28, 29). *E. histolytica* genes contain codons that are very A:T rich (30), whereas mammalian cells prefer more G:C rich codons. Thus to achieve the highest possible expression of protein GL6, we re-synthesized completely its coding sequence, using the codon bias seen in gerbil genes, which is very similar to the one observed in murine genes. Despite several attempts at inserting the wild type sequence encoding GL6 into the pCIST vector, we could never obtain a stable clone with the correct construct; therefore we could not compare the effect of the optimized with the wild-type coding sequences on expression level. However, in the light of the above mentioned reports, we strongly believe that the changes we made to the coding sequence could only have been beneficial. Other features integrated in our DNA vaccine to potentiate protein expression include the use of a strong promoter/enhancer element (hCMV), a chimeric intron upstream of the coding sequence (31), a Kozak consensus sequence (32), and the optimal stop codon and following nucleotide (TGAG) for mammalian termination of translation (33).

The *E. histolytica* Gal-lectin has been described as an immunogenic and antigenically stable molecule (34, 35). It was first considered as a potential subunit

vaccine candidate when the native molecule emulsified in Freund's adjuvant was injected into gerbils and protected the animals at 67% against an intrahepatic challenge with live trophozoites (9). In follow-up studies, different recombinant molecules that included the cysteine-rich region were shown to be as effective, if not more, at conferring protection as the native Gal-lectin: aa 649-1201 administered in Freund's adjuvant had a 81% efficacy (10); aa 767-1138 injected with Titermax as adjuvant conferred protection at 71% (11); and gerbils immunized with aa 945-1059 of the lectin's heavy subunit showed a 62.5% protective efficacy against intrahepatic challenge (13). These studies clearly show that portions of the Gal-lectin are effective at stimulating a protective immune response against amebiasis.

MAbs against the cysteine rich region of the Gal-lectin's heavy subunit have been shown to inhibit adherence of amebae to target cells (21). A carbohydrate-recognition domain (CRD) was mapped to that region; in one report, an *in vitro*-translated protein including aa 352-1139 of the heavy subunit exhibited GalNAc-specific binding activity (36), and it was proposed that residues 352-476 and 986-1139 may contribute to high affinity binding to the sugar. In another report, aa 895-998 produced in *E. coli* as a poly-histidine protein also bound GalNAc with high affinity (37).

In pilot experiments, we constructed DNA vaccines that included wild type sequences encoding larger portions of the Gal-lectin's cysteine-rich region (aa 483-1137 and 754-1137 of Hgl1), but none of them stimulated a significant antiamebic immune response in mice, due to a poor protein expression by transfected cells (data not shown). These proteins, in addition to being encoded by very A:T rich sequences, also contain several cysteine residues, which make them insoluble and difficult for mammalian cells to

produce. Considering the literature cited above, we selected aa 894-1081 (protein GL6) to be included in a new DNA vaccine, since it contains the CRD as previously defined (37), and includes epitopes recognized by two amebic adherence inhibiting MAbs, H85 and 8C12 (21). Moreover, GL6 also includes aa 1005-1029, a 25-mer peptide that was shown to induce a protective humoral immune response in the gerbil model (38). GL6 was mostly insoluble when expressed in COS-7 cells. This may affect the type of immune response induced by the vaccine if the same phenomenon happens *in vivo*; the protein, being expressed mostly intracellularly, may induce a cytotoxic T lymphocyte response more efficiently than a T helper lymphocyte response.

The mechanism of protection from ALA by the Gal-lectin or its portions is not known. While there is a lack of correlation between pre-challenge anti-Gal-lectin antibody levels and protection (9, 11), passive immunization studies suggest that anti-Gal-lectin serum antibodies may play an important role in protection (13, 37, 39). Even though they can be capped (40) or degraded (41) by trophozoites, they are thought to block binding of the parasites to target cells and prevent tissue damage. While pooled sera (1:100) from human patients with ALA, which contain antibodies that recognize a multitude of amebic cell surface proteins, inhibited adherence of trophozoites to CHO cells at 94% in an adherence assay, sera from mice vaccinated with pCISToGL6 showed a 29% inhibition at the same dilution, which is impressive considering that the antibodies in them are directed against a small portion of a single molecule. This effect is also comparable to the 53% inhibition obtained with a 1:100 dilution of sera from immune gerbils injected 3 times with 10 μg native Gal-lectin in Freund's adjuvant (9).

Anti-amebic cell-mediated immunity might also play a role in protection against invasive amebiasis. Gerbils injected with native Gal-lectin developed anti-amebic cell-mediated immunity, also characterized by Th1-type cytokine production by spleen cells and amebicidal activity by nonadherent splenocytes (42), and a reduction in abscess size was achieved without detectable anti-lectin antibodies by delivering the lectin's cysteine rich region orally to gerbils as an attenuated *Salmonella* vaccine (12). It was shown in our laboratory that native Gal-lectin can directly stimulate the Th1-promoting proinflammatory cytokines TNF and IL-12 from macrophages (15, 43), and therefore could act as its own Th1 adjuvant. Adherence-inhibitory MAbs abrogated that activity, suggesting that the CRD was responsible for it. Since protein GL6 includes the Gal-lectin's CRD, it is possible that it can have the same adjuvant effect. This, however, remains to be tested. The pCISToGL6 vaccine induced the generation of Gal-lectin-specific T cells in mice, and the production of anti-Gal-lectin antibodies of the IgG2a isotype, which is an indication of a Th1 type immune response. The cytokine profile expressed by those T cells, the presence or absence of splenocyte amebicidal activity and the efficacy of the vaccine at conferring protection against ALA in the gerbil model are currently under investigation.

Epidemiological studies have shown that presence of anti-Gal-lectin stool sIgA is associated with protection against reinfection with *E. histolytica* (2). An effective amebiasis vaccine should therefore be able to stimulate an adherence inhibitory anti-amebic mucosal immune response. Oral immunization of mice with aa 767-1138 of the heavy subunit together with cholera holotoxin generated an anti-Gal-lectin sIgA intestinal response, and those antibodies showed adherence-inhibitory activity *in vitro* (44). In the

light of the results reported here, the pCISToGL6 vaccine, administered *via* the mucosal route with a proper adjuvant (45), should also be able to induce the production of adherence-inhibitory anti-Gal-lectin sIgA in the gut and confer protection against *E. histolytica* colonization.

ACKNOWLEDGEMENTS

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CONNECTING STATEMENT I

In Manuscript I, we have described the construction of a codon-optimized Gal-lectin-based DNA vaccine. In addition, we have shown that, when administered intradermally to Balb/c mice, this vaccine stimulated a Gal-lectin-specific Th1 immune response. A Th1 (cellular) immune response is believed to be protective against systemic *E. histolytica* infection, as interferon (IFN)-γ and tumor necrosis factor, the main lymphokines produced during such a response, can activate macrophages and make them produce nitric oxide, a potent amebicidal molecule. Interleukin (IL)-12 is a proinflammatory cytokine that acts on T lymphocytes and natural killer cells to make them proliferate and produce IFN-γ and, doing so, plays a central role in the development of a Th1 immune response. To enhance the immunogenicity of the antigen encoded by our DNA vaccine, and to ensure that the vaccine stimulates a strong cellular immune response, we considered the possibility of injecting, together with the DNA vaccine, a mammalian expression vector encoding IL-12. However, since the vaccine was ultimately destined to confer protection in the gerbil model of invasive amebiasis, a plasmid encoding gerbil IL-12 needed to be used. Manuscript II describes the cloning of gerbil IL-12 p40 and p35 subunits, and their expression in mammalian cells as a single-chain, bioactive protein.

MANUSCRIPT II

Molecular cloning of gerbil interleukin-12, and its expression as a bioactive single-chain protein*

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* an abbreviated version of this manuscript was published in the journal

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ABSTRACT

Complementary DNAs coding for gerbil interleukin-12 (IL-12) p40 and p35 subunits were cloned by a combination of cross-species reverse transcriptase polymerase chain reaction (RT-PCR) and 3' rapid amplification of cDNA end (RACE) techniques. IL-12 p40 and p35 were found to have a 79% nucleotide identity and an 81% amino acid similarity to mouse IL-12 p40 and p35, respectively. The p40 and p35 subunits (the latter without its secretion signal peptide) were expressed as a single polypeptide, separated by a short, flexible hinge sequence that allowed for proper folding and assembly. COS-7 cells transfected with a mammalian expression plasmid containing the DNA coding for this single-chain gerbil IL-12 (pSCjIL12) secreted high levels of the protein. The cytokine was bioactive, as it stimulated the proliferation of ConA-activated gerbil spleen lymphoblasts in a dose-dependent manner. Purified single-chain IL-12 and the pSCjIL12 construct could eventually be used in immunological studies, gene therapy or DNA vaccination trials in gerbils.

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*) is currently being used as an animal model for a number of pathological conditions. It is used in studies on cancer (1) and on infectious diseases of viral (2), bacterial (3), but mostly parasitic origin (4, 5). However, most of the work done on gerbils pertains to physiology or pharmacology. Because of a lack of gerbil-specific immunological reagents such as recombinant cytokines and anti-cytokine monoclonal or polyclonal antibodies, there are very few reports of studies focusing on immunological parameters. In these cases, the assays performed relied either on basic phenomena such as lymphoproliferation (6) or macrophage activation (7), or on cross-species bioassays when the gerbil cytokines studied were conserved enough to work on cells from other species, as it is the case for IL-2, IL-4 and TNF-α (6). As studies on the roles of other gerbil cytokines with high species specificity and low cross-reactivity are currently impossible, there is clearly a need to identify gerbil cytokine coding sequences and use them to produce immunological reagents. This improvement in the knowledge of the gerbil immune system will in turn help us to learn more about the diseases gerbils are susceptible to.

Interleukin-12, which was initially named "cytotoxic lymphocyte maturation factor" (8) and "natural killer cell stimulatory factor" (9), is a proinflammatory cytokine that stimulates the proliferation of T cells and natural killer (NK) cells (10), induces IFN-γ production by those cells (11, 12), and enhances the cytolytic activity of cytotoxic T cells and NK cells (9, 13). IL-12 thus plays a critical role in the induction of cell-mediated immune responses, as it promotes the differentiation of naïve, Th0 cells to a Th1

phenotype in the presence of IL-2, while inhibiting the development of Th2 cells, mainly through its IFN-γ production-stimulating activity (14).

IL-12 is composed of a 35-kDa subunit (p35) linked by a disulfide bond to a 40-kDa subunit (p40). The subunits are encoded by two different genes and are therefore differentially regulated; p35 is expressed constitutively by most cell types and can be upregulated (15), while p40 is produced by professional antigen-presenting cells such as macrophages, dendritic cells and B cells, and by neutrophils, making the production of the heterodimeric, bioactive molecule (p70) also restricted to those cells. The p40 subunit is often expressed in excess over p35, in which case it can dimerize to yield a molecule that can bind the IL-12 receptor on target cells, but without triggering any signal (16). p40 homodimers can thus compete with p70 for receptor binding and act as antagonists (17). Herein, we report the cloning and characterization of gerbil IL-12 (p40 and p35), and its *in vitro* expression by transfected cells as a bioactive single-chain protein.

MATERIALS AND METHODS

Collection of cells and RNA

Resident peritoneal cells from 50-60 days old female gerbils (Charles River. St. Constant, Canada) were harvested by lavage of the peritoneal cavity as described (18), with 10 ml of ice-cold RPMI 1640 medium (GIBCO, Burlington, Canada) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 24 mM HEPES (Sigma, St. Louis) and 10% fetal bovine serum (FBS; GIBCO). The erythrocytes were lysed with

Gey's salts (18), and the remaining cells were resuspended in the above medium at a density of 5 x 10^6/ml. The suspension was aliquoted in a 24-well plate (Falcon; Becton Dickinson) at 1.5 ml/well, and the cells were incubated for 24 h at 37°C in a humidified CO_2 atmosphere, with 1 μg/ml lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; Sigma). The stimulated cells were harvested and their RNA isolated with TRIzol reagent (GIBCO), according to the manufacturer's instructions.

cDNA was generated from 2 μg total RNA in a 50-μl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 4 mM dithiothreitol, 1 mM dNTP (GIBCO), 8 U of RNasin ribonuclease inhibitor (Promega Corp., Madison, WI), 100 U of Moloney MuLV reverse transcriptase (GIBCO) and 0.2 μg oligo-dT primer (GIBCO) for regular PCR, or 20 pmol anchor primer 5'-GGC CAC GCG TCG ACT AGT $AC(T)_{17}$-3' for 3'RACE. The reaction was incubated at 37°C for 1 hour, and the enzyme was then inactivated at 95°C for 5 minutes. All subsequent PCRs contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 – 3.5 mM $MgCl_2$, 40 μg dNTP, 50 pmol of both sense and antisense primers, 2.5 U *Taq* DNA polymerase and 5 μl of cDNA, in a total volume of 50 μl. The PCR conditions always consisted of: 30 s at 95°C, 1 min at 55°C, and 2 min at 72°C for 35 cycles, and a final cycle with the extension step at 72°C for 8 min. PCR products were separated by electrophoresis in 1-2% agarose gels containing ethidium bromide. All relevant DNA bands were sliced out, purified using the Sephaglas BandPrep Kit (Amersham Pharmacia Biotech, Baie d'Urfé, Canada), inserted in the cloning vector pGEM-T Easy (Promega), and sequenced.

Cloning of gerbil IL-12

To clone the IL-12 p40 cDNA, PCR was first done with the primers "P40_5' sense": 5'-CAG CTC GCA GCA GAG CAA G-3' and "p40O antisense": 5'-GCC ATG AGC ACG TGA ACC GT-3', which are based on the mouse IL-12 p40 cDNA sequence (GenBank #M86671). The 446 bp product was cloned and its sequence used to design the primer "p40F20 sense": 5'-TAC CTG CCA CAA AGG AGA CCA G-3'. A semi-degenerate primer, "p40_3' antisense": 5'-YTT ARY RTM WTC CAC TTT TC-3', was designed by comparing the mouse and the human (GenBank #M65290) p40 sequences, and used for PCR together with p40F20 sense. A smear was obtained after electrophoresis, and the products of 500-900 bp in length were sliced out, eluted from the gel and ligated to pGEM-T Easy. The ligation reaction was then used as template for PCR using the nested primer "p40S2 sense": 5'-CGT CTG CTG CTC CAC AAG-3' and the T7 sequencing primer 5'-AAT ACG ACT CC TAT AG-3', which binds upstream of the insertion site of pGEM-T Easy. This time, a single product of 798 bp was obtained, inserted in pGEM-T Easy and sequenced from the SP6 site of the vector.

As a first step in cloning of gerbil IL-12 p35 cDNA, PCR was done using the primers "p35_5' sense": 5'-TCC TCT CAG TGC CGG TCC AGC-3' and "p35int antisense": 5'-CTG GTA CAT CTT CAA GTC CTC-3', both based on the mouse IL-12 p35 cDNA (GenBank #M86672). A 405 bp product was obtained and analyzed. Its sequence was used to design the primer "p35F2 sense": 5'-TCA CCC TTG TCC TAA TCC ACC TC-3'. The latter was used in another PCR together with the semi-degenerate primer "p35R4 antisense": 5'-GAR YTC AGA TAG CYC ATC-3', which was based on

the mouse and woodchuck (GenBank #X97018) sequences. A 595 bp product was obtained and its nucleotide sequence determined.

The forward primer "p35F7 sense": 5'-CAG ACT GTG CCC CGG AAA CC-3', based on the known gerbil sequence, was used in 3'RACE together with the adapter primer 5'-GGC CAC GCG TCG ACT AGT AC-3'. The reactions were then diluted 1:10 in water and used as template for a semi-nested PCR with primer "p35F6 sense": 5'-AGA CCC TAT CAA ATT GGA AAT GC-3' (based on the gerbil sequence) and the adapter primer, yielding a 613 bp product that spanned the 3' end of the IL-12 p35 cDNA.

Construction of the single-chain gerbil IL-12-encoding plasmid

The hinge-encoding sequence was generated by mixing 1 μg of each of the two oligonucleotides "Hinge sense": 5'-AA TTC GGA GGA GGA GGC AGC GGC GGC GGA GGA AGC GGC GGA GGC G-3' and "Hinge antisense": 5'-GA TCC GCC TCC GCC GCT TCC TCC GCC GCC GCT GCC TCC TCC TCC G-3' in a buffer containing 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 50 mM NaCl, and annealing the oligonucleotides by heating the reactions to 94°C for 5 minutes and cooling it down to 37°C for 5 minutes. The product was then inserted into the *Eco*RI/*Bam*HI sites of pBluescript II SK+ (Stratagene, La Jolla, CA) to give pHinge.

DNA coding for gerbil IL-12 p35 amino acids 22-210 and the stop codon was amplified by PCR from cDNA derived from LPS-stimulated peritoneal cells, using the primers "scjIL12p35 sense": 5'-CAT GGA TCC AGA GCC AAG CCG GTC TCC GG-3' and "scjIL1235 antisense": 5'-GA TGC GGC CGC TCA GGA GGA GTT CAG ATA GC-3', and *Pwo* DNA polymerase (Boehringer Mannheim, Laval, Canada), according to

the manufacturer's instructions. The product was digested and ligated into *Bam*HI/*Not*I-treated pHinge, to yield pHingep35.

Meanwhile, the gerbil IL-12 p40 coding sequence (without the stop codon) was generated as for the p35 sequence, but with the PCR primers "scjIL12p40 sense" 5'-CTA CTC GAG CTC GCA GCA GAG CAA GAT GTG-3' and "scjIL12p40 antisense": 5'-ACT GAA TTC TTT GCA GGG CAC GCA CGC ATG-3'. This product was digested and ligated into the *Xho*I/*Eco*RI sites of the mammalian expression vector pCI-neo (Promega), to give pCI-neop40. Finally, pHingep35 was treated with *Eco*RI and *Not*I, and the fragment encoding the hinge sequence fused to the p35 subunit was inserted into *Eco*RI/*Not*I-treated pCI-neop40 to yield the final construct pSCjIL12. The whole insert in this plasmid was sequenced to make sure there were no mistakes in the hinge portion and that the subunit sequences matched perfectly those found in the initial cloning steps.

Expression of gerbil IL-12 p35 in* E. coli*, purification and generation of antiserum

To facilitate the purification of recombinant gerbil IL-12 p35 protein, the latter was expressed in *E. coli* as a fusion protein with an N-terminus hexa-histidine tag. To make the plasmid construct, the IL-12 p35 coding sequence was cut out of pHingep35 with *Bam*HI and *Sac*I and inserted into the bacterial expression vector pQE-30 (Qiagen, Toronto, Canada) previously digested with the same restriction enzymes. The resulting plasmid, pQE-30p35, was used to transform *E. coli* TOP10F'.

A culture of TOP10F'pQE-30p35 was grown for 12 hours at 37°C with shaking, in 250 ml LB broth supplemented with 100 μg/ml ampicillin, and the cells were harvested by centrifugation. The cell pellet was resuspended in 20 ml binding buffer (20 mM Tris-

HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole) supplemented with 50 mg lysozyme, incubated at 37°C for 1-2 hours and sonicated. The lysate was then ultracentrifuged at 40 000 x *g* for 30 minutes at 4°C, and the cell pellet was dissolved in binding buffer containing 8 M urea. After an overnight incubation at room temperature, the extract was sonicated, ultracentrifuged as above, and the supernatant applied to a Ni-NTA superflow column (Qiagen) equilibrated with the urea-containing binding buffer. The column was washed with wash buffer made of 20 mM Tris-HCl, pH 7.9, 8 M urea, 0.5 M NaCl, 0.5% Tween-20 and 60 mM imidazole, and the bound proteins were eluted with elution buffer containing 20 mM Tris-HCl, pH 7.9, 8 M urea, 0.5 M NaCl, and 0.5 M imidazole. The eluate was dialyzed against distilled water, lyophilized, and the remaining proteins dissolved in 1 ml phosphate-buffered saline. Protein concentration in the sample was determined with the BCA protein assay kit (Pierce, Rockford, IL), using bovine serum albumin (Sigma) as standard.

Balb/c mice (Charles River) were injected subcutaneously three times, two weeks apart, with 10 μg of the purified recombinant gerbil IL-12 p35 emulsified in complete (first injection) or incomplete (second and third injections) Freund's adjuvant (ICN, Aurora, OH). The animals were bled 10 days after the last injection and their sera were collected and pooled.

Expression of SCjIL12 in COS-7 cells

COS-7 cells were plated at a density of 4 x 10^5/ml in 60-mm culture dishes (Sarstedt, Newton, NC) in DMEM (GIBCO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 24 mM HEPES and 10% FBS, and incubated overnight. They

were then transfected with 1 μg of CsCl gradient centrifugation-purified pSCjIL12 or pCI-neo (control vector), using LipofectAmine reagent (GIBCO) in serum-free, antibiotic-free DMEM. After a 5-hours incubation in the presence of the DNA-liposome complexes, the medium was removed and replaced with DMEM containing antibiotics but no serum. The cultures were incubated for another 72 hours, and the cells and their supernatants were harvested. After centrifugation at 300 x *g* for 10 minutes, the supernatants were either concentrated 20:1 by lyophilization or saved and kept at 4°C, while the cell pellets were dissolved in 50 μl of reducing SDS-PAGE sample buffer (19) and boiled for 5 minutes.

Immunoblot analysis of SCjIL12 expression by transfected COS-7 cells

Cells, supernatants and purified recombinant gerbil IL-12 p35 were separated in a 10% SDS-polyacrylamide gel under denaturing conditions, and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was probed with a 1:500 dilution of the mouse anti-gerbil IL-12 p35 antiserum, washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse Ig antibody. Peroxidase activity on the membrane was visualized using the enhanced chemiluminescence system (ECL; Amersham).

Proliferation-based IL-12 bioassay

This assay is based on the ability of ConA-activated lymphoblasts to proliferate in the presence of IL-12 (20). The spleen from a 50-60 days old female gerbil was homogenized and the erythrocytes were lysed. Splenocytes were resuspended at a density

of 2 x 10^6/ml in RPMI 1640 medium containing HEPES, penicillin, streptomycin (see above), 2% FBS and 2 μg/ml ConA (Sigma). The medium also contained 7% conditioned medium from COS-7 cells expressing gerbil IL-2. The suspension was distributed in a 24-well plate (2 ml/well) and incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere. The lymphoblasts were then harvested, washed twice in RPMI, resuspended in RPMI + 5% FBS at a density of 1 x 10^6/ml and plated in a 96-well culture plate (Falcon) (50 μl/well). An equal volume of 1:10 serially diluted COS-7 supernatant (control or containing single-chain gerbil IL-12) was added to the cells; each condition was done in triplicates. The cultures were incubated for 24 hours, and 1 μCi [^{3}H]thymidine in RPMI + 5% FBS was added to each well in a volume of 50 μl. The cells were incubated overnight and harvested onto glass fiber filters, using an automated cell harvester (Skatron, Lier, Norway). The radioactivity incorporated in the cells was finally measured by scintillation counting (LKB Wallac; Pharmacia).

RESULTS

Nucleotide and amino acid sequences of gerbil IL-12 p40 and p35

Using the cross-species RT-PCR and 3'RACE techniques, we have determined the cDNA sequences for gerbil IL-12 p40 and p35 (Fig. 1A and B). The gerbil IL-12 p40 sequence (Fig.1A) starts with the initiation codon and includes a 1035-bp open reading frame (ORF) that is 79% identical to its mouse counterpart, and a short portion of the 3' untranslated region (UTR). The polypeptide encoded by the ORF contains 344 amino

A

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                    20                  40                  60                  80                 100                 120
ATGTGTCATCAGAAGCTAACCATCTCCTGGTTTGCTGTGGTTCTGCTGGCATCTCCACTTATGGCCATATGGGAGCTGGAGAAAAATGTTTATGTTGTAGAGATGGACTGGAGGAGCGGT
 M  C  H  Q  K  L  T  I  S  W  F  A  V  V  L  L  A  S  P  L  M  A  I  W  E  L  E  K  N  V  Y  V  V  E  M  D  W  R  S  G

                   140                 160                 180                 200                 220                 240
GCGAATGAAAGCGTGGTCCTCACCTGTGACACTTCTGAAGAGGGTGATATCACCTGGACCAAAGACCAGACGACCAAAGTAGAGCAATCTGGAAAAACCGTGACCATCGAAGTCAAAGAC
 A  N  E  S  V  V  L  T  C  D  T  S  E  E  G  D  I  T  W  T  K  D  Q  T  T  K  V  E  Q  S  G  K  T  V  T  I  E  V  K  D

                   260                 280                 300                 320                 340                 360
TTCAATGAAGCTGGTCAATATACCTGCCACAAAGGAGACCAGACCCTCAGCCACTTCCGTCTGCTGCTCCACAAGAAGAAAAACGGATATTGGTCCAATGAGATCTTAAAGCAGGATAAA
 F  N  E  A  G  Q  Y  T  C  H  K  G  D  Q  T  L  S  H  F  R  L  L  L  H  K  K  K  N  G  Y  W  S  N  E  I  L  K  Q  D  K

                   380                 400                 420                 440                 460                 480
AATTCCGAAAATCAGACTTTCCTGAAATGTGAGGCAGCAAATTACTCCGGGCGTTTCACGTGCTCTTGGCTGGTGGAAAGGACTGACTTGAAGTTCAGCATCAAGAACAGTAGTAGTTCC
 N  S  E  N  Q  T  F  L  K  C  E  A  A  N  Y  S  G  R  F  T  C  S  W  L  V  E  R  T  D  L  K  F  S  I  K  N  S  S  S  S

                   500                 520                 540                 560                 580                 600
CCTGACTCCCGAGCAGTGACGTGTGGAGCTGTGTCTCTGACCGCAGAGAAAGTCAAAGGGGATGAAAAGGATGGTAAGCTTAAGCAGTACTCAGTGACGTGCCAGGAGGACATCACCTGC
 P  D  S  R  A  V  T  C  G  A  V  S  L  T  A  E  K  V  K  G  D  E  K  D  G  K  L  K  Q  Y  S  V  T  C  Q  E  D  I  T  C

                   620                 640                 660                 680                 700                 720
CCAACTGCAGAGGAGACTGTGCCCATTGGCCTGGTGCTGGAGGCACAGGAGCAGAATAAATATGAGAACTACAGCACCAGCTTCTTCATCAGAGACATCATCACACCGGACCCTCCCAAG
 P  T  A  E  E  T  V  P  I  G  L  V  L  E  A  Q  E  Q  N  K  Y  E  N  Y  S  T  S  F  F  I  R  D  I  I  T  P  D  P  P  K

                   740                 760                 780                 800                 820                 840
AACCTGAAGGTGAAACCTTTGAAGAACGATAAGGTGGAGGTCAGCTGGGAGTACCCTGACTCCTGGAGCACCCCCCATTCCTACTTCGCCCTCAAGTTCTATGTTTGGACCGAGAGCAAT
 N  L  K  V  K  P  L  K  N  D  K  V  E  V  S  W  E  Y  P  D  S  W  S  T  P  H  S  Y  F  A  L  K  F  Y  V  W  T  E  S  N

                   860                 880                 900                 920                 940                 960
AAAGAAAATAAGATGAGGAACACAAATAGCAATAAAGAAAATAAGATGAGGAACGCATGTATACAGAATGGCACATGGCTAGTAGACACGACCTCCACCAAAGTCCGGTGCAAAGATGGA
 K  E  N  K  M  R  N  T  N  S  N  K  E  N  K  M  R  N  A  C  I  Q  N  G  T  W  L  V  D  T  T  S  T  K  V  R  C  K  D  G

                   980                1000                1030                1040                1060
AGAGTCTGCGTGGAGGCTCAGGATCGCTACTACAATTCTTTGTATAGCAAACATGCGTGCGTGCCCTGCAAATAGGGTCCAGTCCCAGAATGCAACTTTGGA
 R  V  C  V  E  A  Q  D  R  Y  Y  N  S  L  Y  S  K  H  A  C  V  P  C  K  *
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B

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                    20                  40                  60                  80                 100                 120
ATGCGTCAGTCACGCCGCCTCTTCTTTTTGGTCACCCTTGTCCTAATCCACCTCAGTTTGGCCAGAGCCAAGCCGGTCTCCGGACCCGCCCGGTGTCTTAACAGCTCCCAAACCCTGCTC
 M  R  Q  S  R  R  L  F  F  L  V  T  L  V  L  I  H  L  S  L  A  R  A  K  P  V  S  G  P  A  R  C  L  N  S  S  Q  T  L  L

                   140                 160                 180                 200                 220                 240
AGGACTACGGACAACGCGCTGAAGACGGCCAGACAAAAGCTAACTAACTACTGTGGTGCTGAAGACTTCGATCGCGAAGACATCACAAAAGACAAAACCAGCACACTGAAGGCCTGTTTA
 R  T  T  D  N  A  L  K  T  A  R  Q  K  L  T  N  Y  C  G  A  A  D  F  D  R  E  D  I  T  K  D  K  T  S  T  L  K  A  C  L

                   260                 280                 300                 320                 340                 360
CCACTGGAACTGGTCAAGAATGAGAGTTGCCTGGCTGGAGAGACTTCTTCCATGATCAGAGGGAGCTGCCTGCTTCCCCAAAAGACTTCTTCCATGATGACCCTGTGCCTTAGTAGCATC
 P  L  E  L  V  K  N  E  S  C  L  A  G  E  T  S  S  M  I  R  G  S  C  L  L  P  Q  K  T  S  S  M  M  T  L  C  L  S  S  I

                   380                 400                 420                 440                 460                 480
TATGAGGACTTGAAGATTTACCAGACAGAGTTCCAGGCCATAGACGCAGAGCTTCGGCGTCAGAATCAGATCACTCTGGACAAGGACATGTTGGCAGCCATTGATGAGCTGATGCAGGCT
 Y  E  D  L  K  I  Y  Q  T  E  F  Q  A  I  D  A  E  L  R  R  Q  N  Q  I  T  L  D  K  D  M  L  A  A  I  D  E  L  M  Q  A

                   500                 520                 540                 560                 580                 600
CTGAATCATAATGGGCAGACTGTGCCCCGGAAACCTTACCTGGCAGAAAGAGACCCTATCAAATTGGAAATGCATCTCTGCATTCAACTCCATGCTTTCAGCATCCGAGTCACGACCATC
 L  N  H  N  G  Q  T  V  P  R  K  P  Y  L  A  E  R  D  P  I  K  L  E  M  H  L  C  I  Q  L  H  A  F  S  I  R  V  T  T  I

                   620                 640                 660                 680                 700                 720
AACAAAGTGATGAGCTATCTGAACTCCTCCTGAAAGGCCCCAGTCCCTCCCCTTTAGTGCCCTCTTTGCAAAACTATGAACCAAAGAAATTTTAATACGAGGTGGGCTAAGAGTCAGGAA
 N  K  V  M  S  Y  L  N  S  S  *

                   740                 760                 780                 800                 820                 840
ACCTTAGCCTAGTTGACCTTAAGTTCCTGCAGAAACCGATGCTCCTATCACATTCCCACCCCCCATCAACGGTGGCACAGCTACCTCAGCACGGCCCCTTCAGTCATGTCTCATATTTAT

                   860                 880                 900                 920                 940                 960
TATGCACGTTGTTTGTAAGAAAATGTCCTCCCTGAAAGTGTCGGCAGAAGGGCAAGATCTGATAAGCTAGTCTTTTTGTACCAAAGTGTTTATGAAAACACTCAGTCACCACTTATTTAA

                   980                1000                1020                1040                1060                1080
AAATATTTATTGTTATATTTTATATTCATGAAAGTATATGAGCTTATTTATATTTATTTATGTACTATTTATTATAATATTTCTTATAAATCAATTTGAAACATAACTTATGTGGTTTTA

                  1100
ATAAAGTGATGCAAGCACTTATAAAAAAAAAAAAAAAAA
```

Figure 1. Nucleotide sequences and corresponding amino acid sequences of gerbil IL-12 p40 (A) and p35 (B) cDNAs. The nucleotide positions are numbered above the DNA sequences, and an asterisk identifies the stop codon. For the p35 sequence, the ATTTA mRNA destabilizing motifs are underlined, and the consensus poly(A) addition site is shown in bold characters. The GenBank accession numbers are AF288612 for gerbil IL-12 p40, and AF288849 for gerbil IL-12 p35.

acids, the first 22 making up the putative secretion signal peptide (based on the human protein (21)). The mature p40 protein has a calculated molecular weight of 36.9 kDa. It has 12 cysteine residues which are all conserved in the mouse p40 subunit (Fig. 2A), including the one forming an intermolecular disulfide bridge in the formation of p40 homodimers (C^{197} in the mouse (22), C^{200} in the gerbil); however, the mouse protein has an additional cysteine residue that gerbil p40 lacks. Gerbil p40 also has 6 potential N-glycosylation sites, of which only three are conserved in the mouse counterpart. Overall, gerbil IL-12 p40 is 81% similar to the mouse protein at the amino acid level.

The 1120-bp gerbil IL-12 p35 cDNA sequence and its predicted translation product are shown in Figure 1B. The DNA sequence starts with the initiation codon and includes a 633-bp ORF, as well as a 3'UTR that extends to the polyA tail. This 3'UTR contains 7 ATTTA mRNA destabilizing sequences and a poly-adenylation signal sequence AATAAA. The ORF, which is 79% identical to the one coding for mouse p35, encodes a 210-aa precursor polypeptide with an amino-terminal 21-aa secretion signal sequence (based on the human p35 protein (21)). The mature gerbil p35 protein has a calculated molecular weight of 21.2 kDa and has 7 cysteine residues, which are all conserved in mature mouse p35 (Fig. 2B). It also has 3 potential N-linked glycosylation sites, whereas mouse p35 only has a single one, which is conserved in the gerbil protein. The three leucine-zipper motifs found in human, mouse and cat p35 amino acid sequences (23) are also conserved in the gerbil sequence. The amino acid similarity between gerbil and mouse p35 is 81%.

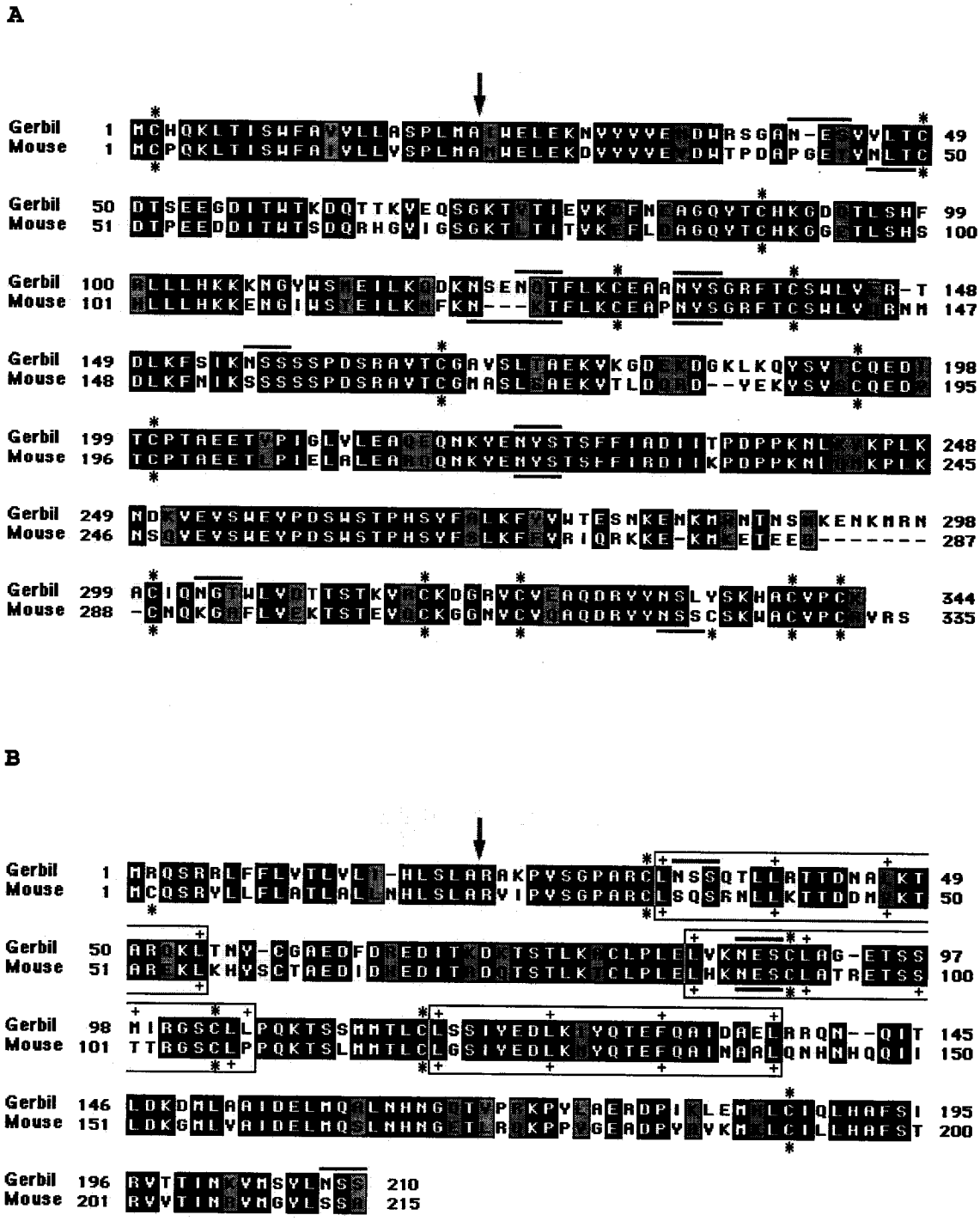

Figure 2. Alignment of the deduced amino acid sequences of gerbil and mouse IL-12 p40 (A) and p35 (B) proteins. The amino acid residues are numbered at the beginning and the end of each line of the alignments. Conserved residues are boxed in black and similar residues in light grey. The arrows show the putative signal peptide cleavage sites, asterisks indicate cysteine residues, and potential N-linked glycosylation sites are over- or underlined. For the p35 sequences, the three leucine zipper motifs are boxed, with their characteristic leucine residues (or substitutes) marked by plus (+) signs.

Construction of the SCjIL-12 mammalian expression plasmid

The coding sequences for gerbil IL-12 p40 and p35 were amplified using the primers based on the gerbil sequences, except for the "scjIL12p40 sense" primer, which was based on the 5'UTR of mouse IL-12 p40 cDNA. The stop codon of the p40 sequence was replaced with an *Eco*RI site to allow fusion with the hinge sequence (Fig. 3). Because of the addition of this restriction site, two foreign amino acids (Glu and Phe) are found between the p40 and hinge translated sequences; however, their presence does not disrupt the bioactivity of the single-chain IL-12 (see below). The hinge sequence was designed using the two most frequent codons for glycine (GGC and GGA) and serine (AGC and TCC) seen in gerbil coding sequences. The last Gly and Ser residues of the hinge sequence are encoded by GGA and TCC, respectively, which also make up the *Bam*HI site used for the fusion with the p35 coding sequence without the insertion of foreign amino acids. The p35 sequence encodes the mature form of the protein (without the secretion signal peptide sequence). The SCjIL-12 coding sequence was inserted in the pCI-neo vector to allow a high expression of the protein in mammalian cells. The calculated molecular weight of the mature single-chain gerbil IL-12 is 59.4 kDa.

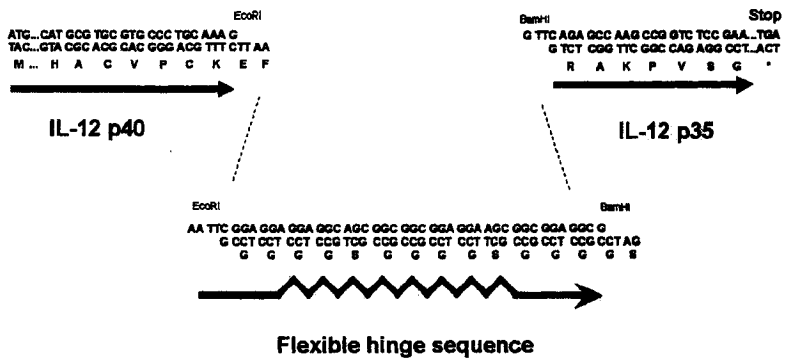

Figure 3. Nucleotide and amino acid sequences of the flexible hinge sequence linking the IL-12 p40 and p35 subunits in the SCjIL12 protein. The restriction sites used for the construction are indicated at the junctions.

Expression of SCjIL-12 in COS-7 cells and immunoblot analysis

COS-7 cells transfected with pSCjIL-12 (or pCI-neo as control) for 72 hours, and their concentrated supernatants, were analyzed by immunoblot using antiserum against recombinant gerbil p35 (Fig. 4). A single immunoreactive protein at 80 kDa could be detected in the supernatant from cells transfected with pSCjIL-12, but not in the control supernatant. The same band was also detected in the lysate of pSCjIL-12-transfected cells but not in the control cell lysate. COS-7 cells transfected with pSCjIL-12 produced high levels of secreted single-chain gerbil IL-12.

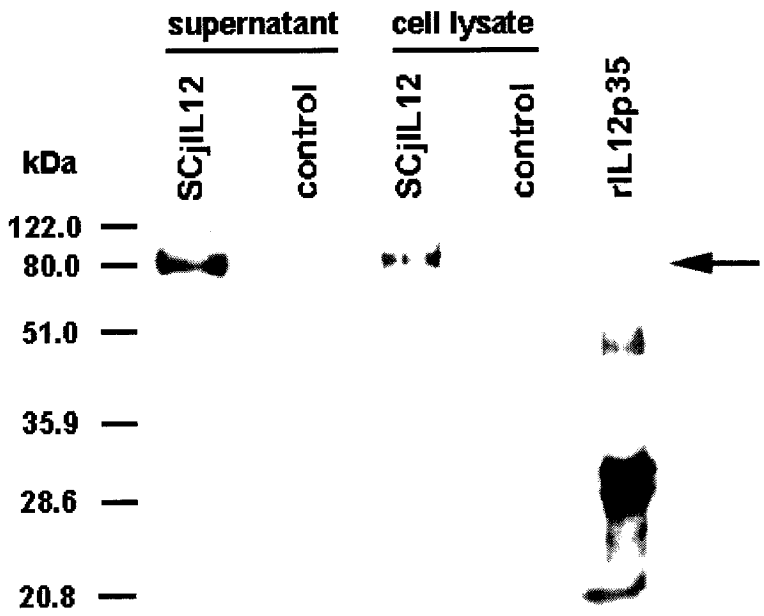

Figure 4. Immunoblot of SCjIL12 produced by COS-7 cells. Aliquots of concentrated supernatants and lysates of COS-7 cells transfected with pSCjIL12 or pCI-neo were separated in a 10% SDS-polyacrylamide gel under reducing conditions. Recombinant gerbil IL-12 p35 (1 μg) was also electrophoresed. The proteins were transferred onto a nitrocellulose membrane and probed with mouse anti-gerbil IL-12 p35 serum. The arrow points to the 80-kDa immunoreactive SCjIL12 protein.

Gerbil IL-12 bioassay

To determine whether COS-7 cells transfected with pSCjIL-12 produced bioactive single-chain gerbil IL-12, serial dilutions of 72-h culture supernatants were tested in a lymphoblast proliferation assay. As shown in Figure 5, the supernatant induced the proliferation of gerbil spleen lymphoblasts in a dose-dependent manner. The highest proliferation was obtained with dilutions of 1:20-1:200, and activity was completely titrated out by diluting the conditioned medium 1:20000. In contrast, supernatants from COS-7 cells transfected with the control plasmid pCI-neo did not stimulate cell proliferation even at a 1:20 dilution.

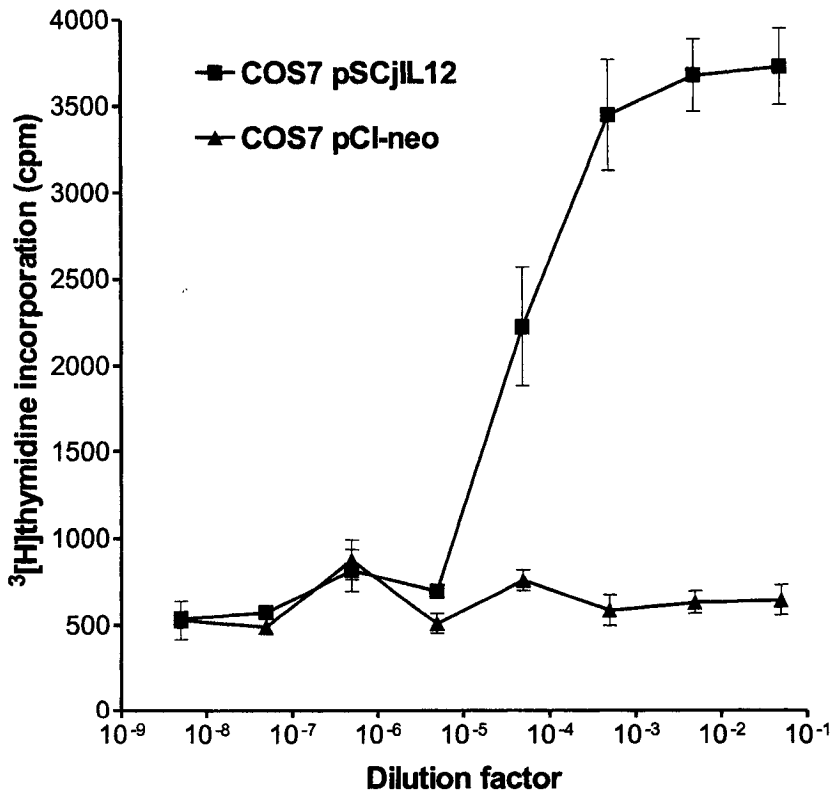

Figure 5. Bioactivity of SCjIL12. Gerbil spleen ConA-activated lymphoblasts were incubated with 1:10 serially diluted supernatants from a 72-hour culture of COS-7 cells transfected with pSCjIL12 or with the empty vector pCI-neo. Incorporated [^{3}H]thymidine was then determined as an indication of cell proliferation. Results are expressed as mean cpm of triplicate cultures ± SE.

DISCUSSION

Herein we report the cloning and expression of gerbil interleukin 12. The cDNAs coding for the p40 and p35 subunits were cloned by RT-PCR, using primers based on known, homologous sequences from other species, and by 3'RACE. mRNA from LPS-stimulated peritoneal cells was used as template. Both gerbil p40 and p35 were found to have a 79% nucleotide identity and an 81% amino acid similarity to mouse IL-12 p40 and p35, respectively. All the cysteine residues in both subunits are conserved in the mouse counterparts. Gerbil p35 has 3 leucine zipper motifs in its amino acid sequence; these motifs are found in mouse p35 as well, but also in the human, feline (23) and woodchuck proteins. Proteins with leucine zippers are able to form dimers with other leucine zipper proteins through the interdigitation of their leucine repeat helices (24). The fact that those motifs remained highly conserved in IL-12 p35 may suggest that they play an important role in that protein. However, to date, there are no reports of p35 subunits forming homodimers or interacting with other leucine zipper-bearing proteins. The significance of the leucine zippers in IL-12 p35 is still unknown.

IL-12 p40 subunits can form homodimers if they are overexpressed compared to the p35 subunit. These heterodimers can bind to the IL-12 receptor but do not lead to its activation, and as such, act as IL-12 heterodimer antagonists, inhibiting the action of heterodimeric IL-12 *in vitro* (16, 17, 25) and *in vivo* (26). If an IL-12 expression vector is to be used to transfect mammalian cells, it is important to avoid overexpression of p40 and simultaneous expression of heterodimeric IL-12 and homodimeric p40. To solve this problem, we expressed gerbil IL-12 as a single-chain protein, which always has the two subunits produced in a 1:1 ratio. The structure of our SCjIL12 was similar to other single-

chain IL-12 proteins already published (27-31). The p40 subunit was placed at the amino end of the protein, while the p35 subunit was placed at the carboxyl end; this order was shown to be optimal, as single-chain IL-12 proteins having the p35 subunit at the amino terminus show very little bioactivity (28). The two IL-12 subunits were separated by the flexible hinge sequence $(Gly_4Ser)_3$ (32), which allows proper folding of the subunits and their subsequent correct assembly into a bioactive protein. As SCjIL12 was destined to be expressed in mammalian cells, the DNA coding for the hinge sequence was synthesized using a mammalian bias (GGC and GGA for glycine, AGC and TCC for serine).

COS-7 cells transiently transfected with a mammalian expression vector bearing the SCjIL12-encoding sequence expressed high levels of a protein that immunoreacted with serum raised against recombinant gerbil IL-12 p35. Although the calculated molecular weight of SCjIL12 is 59.3 kDa, the protein expressed by COS-7 cells had an apparent molecular weight of about 80 kDa. This difference is most likely due to glycosylation of the protein, SCjIL12 containing a total of 9 consensus N-glycosylation sequences. Single-chain gerbil IL-12 was bioactive, as supernatants from pSCjIL-12-transfected COS-7 cells, but not supernatants from cells transfected with the control plasmid, stimulated the proliferation of gerbil ConA lymphoblasts in a dose-dependent manner. Interestingly, SCjIL12 showed no bioactivity on mouse ConA lymphoblasts (data not shown), suggesting that gerbil IL-12 has some species specificity. It was shown that, although mouse IL-12 was bioactive on human PHA blasts, human IL-12 did not stimulate proliferation of mouse ConA blasts (20). Later, another study identified 5 amino acid residues within the mouse p35 protein (K^{57}, H^{58}, S^{60}, H^{146}, P^{184}) which are essential for proper interaction with the IL-12 receptor and signal transduction (33). Those amino

acids are not conserved in human IL-12 p35, which explains the lack of bioactivity of human IL-12 on mouse cells. Similarly, only 1 of the 5 residues (P^{184}) is conserved in gerbil p35 (P^{179}); this might not be sufficient to allow gerbil IL-12 to bind to the mouse IL-12 receptor and/or activate it.

The cloning of gerbil IL-12 will now allow the generation of gerbil-specific immunological reagents (recombinant IL-12, monoclonal/polyclonal antibodies, probes for RNAse protection assay or northern blot, primers for RT-PCR) to study the expression and the role of this cytokine *in vivo* or *in vitro* in different pathological conditions. Moreover, the SCjIL12-encoding mammalian expression vector constructed for this study (pSCjIL12) could be used in gerbil in gene therapy studies or DNA vaccination trials as a Th1 adjuvant.

ACKNOWLEDGEMENTS

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CONNECTING STATEMENT II

Manuscript II reports the cloning of gerbil interleukin (IL)-12, a cytokine that plays a critical role in the development of a cellular (Th1) immune response, due to its strong interferon (IFN)-γ-inducing activity. IL-18, also known as IFN-γ-inducing factor, is another proinflammatory cytokine that is involved in cellular immune responses. Although IL-18 does not drive Th1 development by itself, it can nonetheless stimulate IFN-γ production by T cells, and synergize with IL-12 for an even higher IFN-γ expression by those cells. IL-18 is thus a logical choice of genetic adjuvant to be used with a DNA vaccine that must stimulate an antigen-specific Th1 immune response, such as the Gal-lectin-based DNA vaccine described in Manuscript I. In order to use this strategy in the gerbil model of amebiasis, we cloned the gerbil IL-18 cDNA, and this is reported in Manuscript III, along with the cloning and characterization of the gerbil protease that activates IL-18, caspase-1.

MANUSCRIPT III

Gerbil interleukin-18 and caspase-1: cloning, expression and characterization*

Denis Gaucher and Kris Chadee

* this manuscript has been accepted for publication in the journal

Gene

ABSTRACT

We are reporting the molecular cloning of gerbil interleukin-18 (IL-18) and caspase-1. The cDNAs encoding the molecules were cloned by cross-species reverse transcriptase polymerase chain reaction (RT-PCR) and 5'/3' rapid amplification of cDNA ends (RACE). COS-7 cells transfected with plasmids encoding pro-IL-18 and caspase-1 precursor expressed the two proteins intracellularly. When the cells were lysed in the presence of dithiothreitol, caspase-1 precursor became active and converted pro-IL-18 into mature IL-18. A partially purified preparation of gerbil mature IL-18 was bioactive, as it stimulated the proliferation of gerbil spleen cells in a dose-dependent manner.

INTRODUCTION

Interleukin-18, or interferon (IFN)-γ inducing factor, is a pleiotropic proinflammatory cytokine produced by a wide range of cells, such as macrophages/monocytes (1), dendritic cells (2), Kupffer cells (3), astrocytes and microglia (4, 5), osteoblasts (6) and keratinocytes (7). Although it does not drive Th1 development by itself as IL-12 does (8), it was found to induce high levels of IFN-γ production by T cells independently of IL-12 (9, 10). IL-18 upregulates perforin-mediated cytotoxicity by natural killer (NK) (11) and NK-T cells (12), and Fas-ligand-dependent cell killing by NK cells (13) and Th1 cells (14). The IFN-γ gene promoter was found to be differentially regulated by IL-18 and IL-12 (15), explaining how the latter cytokines can synergize for enhanced IFN-γ production (16, 17). In addition, IL-12 upregulates IL-18 receptor expression (18, 19), while IL-18 enhances IL-12 receptor β2 subunit production (20); one cytokine may thus enhance cell responsiveness to the other.

Through their IFN-γ-inducing activities, IL-18 and IL-12 together contribute to the development of a Th1 response and the suppression of a Th2 response; this is evidenced by an increase in IgG2a accompanied by a decrease in IgG1 and IgE production by activated murine B cells incubated in the presence of both cytokines (21). However, IL-18 in combination with IL-2 was found to induce IL-13 production from NK and T cells (22), and to stimulate IL-4 and histamine release by basophils when used together with IL-3 (23). IL-18 can thus enhance Th1 or Th2 responses, depending on the cytokines that are present at its site of action.

Structurally, IL-18 is very closely related to IL-1; it has the same all β-pleated structure (24), and the binding subunit of its receptor was previously known as IL-1 receptor-related protein (25, 26). Like IL-1β, IL-18 is produced intracellularly as an inactive precursor that must be cleaved to the mature form, most commonly with caspase-1, in order to be activated and secreted (3, 27).

Caspase-1, also known as IL-1β converting enzyme, is a cysteine proteinase produced by several cell types, including macrophages, neutrophils and B lymphocytes (28, 29). Caspase-1 is expressed as a precursor protein that must be cleaved in order to become an enzymatically active heterodimer composed of a 10 kDa and a 20 kDa chain (30). This activation is carried out by another proteinase, caspase-11 (31), or autoproteolytically after oligomerization of caspase-1 (32, 33). Two molecules of caspase-1 heterodimer form a tetramer with 2 molecules of substrate (pro IL-1β or pro-IL-18), prior to processing of the substrate (34, 35).

Because of its susceptibility to infection with a wide range of pathogens, the Mongolian gerbil (*Meriones unguiculatus*) is an attractive alternative animal model to study diseases that cannot affect conventional laboratory animals such as mice, rats, guinea pigs and rabbits, and is indeed increasingly popular as such. However, the lack of commercially available gerbil-specific reagents, such as recombinant cytokines and monoclonal antibodies, has made immunological studies in the gerbil difficult. To correct this situation, we (36) and others (37) have already published cDNA sequences encoding gerbil cytokines. We are now reporting the cloning and characterization of gerbil IL-18 and caspase-1.

MATERIALS AND METHODS

Collection of peritoneal cells and their RNA

Resident peritoneal cells from 50-60 days old female gerbils (Charles River, St. Constant, Canada) were obtained by lavage of the peritoneal cavity, as previously described (38), with 10 ml of ice-cold complete RPMI (cRPMI), made of RPMI 1640 medium (GIBCO, Burlington, Canada) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 24 mM HEPES (Sigma, St. Louis) and 10% fetal bovine serum (FBS; GIBCO). The cells were washed, distributed in 24-well plates ($7.5x10^6$ cells per well) in cRPMI, and stimulated with 1 µg/ml lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; Sigma) for 24 h at 37°C in a humidified 5% CO_2 atmosphere. The stimulated cells were harvested and their RNA isolated using TRIzol reagent (GIBCO), according to the manufacturer's instructions.

cDNA was generated from 2 µg total RNA in a 50-µl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 4 mM dithiothreitol, 1 mM dNTP (GIBCO), 8 U of RNasin ribonuclease inhibitor (Promega Corp., Madison, WI), 100 U of Moloney MuLV reverse transcriptase (GIBCO) and 20 pmol oligo-dT primer, anchor primer or gene-specific primer (see below for details). The reaction was incubated at 37°C for 1 h, and the enzyme was then inactivated at 95°C for 5 min. All subsequent PCRs contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 - 3.5 mM $MgCl_2$, 40 µM dNTP, 50 pmol of both sense and antisense primers, 2.5 U *Taq* DNA polymerase and 5 µl of cDNA, in a total volume of 50 µl. The PCR conditions always consisted of: 30 s at 95°C, 1 min at 55°C, and 2 min at 72°C for 35 cycles, and a final cycle with the

extension step at 72°C for 8 min. PCR products were separated by electrophoresis in 1-2% agarose gels containing ethidium bromide. All relevant DNA bands were sliced out, purified using the Sephaglas BandPrep Kit (Amersham Pharmacia Biotech, Baie d'Urfé, Canada), inserted in the cloning vector pGEM-T Easy (Promega), and sequenced.

Cloning of gerbil IL-18 and caspase-1 cDNAs

To clone the gerbil IL-18 cDNA, two primers were derived from the mouse IL-18 sequence (GenBank accession # NM_008360): "IL-18_5' sense": 5'-CCA GGA ACA ATG GCT GCC-3', based on the region overlapping the start codon, and "IL18_3' antisense": 5'-TAC TAA TCG TCT TTC TGG-3', based on a sequence in the 3' untranslated region (UTR), and PCR was done with them, using as template cDNA reverse transcribed with "IL18_3' antisense". The 630-bp product was cloned and its sequence used to design the primers "jIL-18GSP1 antisense": 5'-ATT TCG TAT CAC CGT GGC TG-3' and "jIL-18 GSP2 antisense": 5'-AGT GAA CAT TAC AGA TTT ATC CC-3'. Finally, 5' RACE was performed using cDNA reverse transcribed with "jIL-18 GSP2 antisense". Briefly, the cDNA reaction was treated with RNAse H, passed through a GlassMax column (GIBCO) to remove the remaining RNA fragments, and a poly(C) tail was added to the cDNA molecules using terminal deoxyribosyl transferase, as recommended by the manufacturer (GIBCO). PCR was done using the tailed cDNA as template, the 5'RACE anchor primer 5'-CGC GTT CAT GTA GCA TCA GGG IIG GGI IGG GII G-3' and "jIL-18 GSP1". A product of 487 bp was obtained, cloned and sequenced.

To clone the cDNA encoding gerbil caspase-1, PCR was done using the primer "mICE_5' sense": 5'-GCG TGT AGA AAA GAA ACG CC-3', based on the region of mouse caspase-1 cDNA that is immediately upstream of the start codon, and "mICE_int antisense": 5'-TCC YGG GAA GAG GTA GAA AC-3', also based on the mouse sequence (GenBank # L28095). The 1223-bp product, which made up most of the coding sequence of gerbil caspase-1, was cloned, sequenced, and used to generate the primer "jICE int sense": 5'-CCA CTC GTA CAC GTC TTG C-3'. A 3'RACE reaction was performed, using cDNA reverse-transcribed with the anchor primer 5'-GGC CAC GCG TCG ACT AGT AC(T)-3', the primer "jICE int sense", and the adapter primer 5'-GGC CAC GCG TCG ACT AGT AC-3'. A product of 841 bp was obtained, cloned and sequenced.

Expression of gerbil IL-18 in* E. coli, *purification and generation of antiserum

To facilitate the purification of recombinant gerbil IL-18 protein, the latter was expressed in *E. coli* as a fusion protein with an N-terminus hexa-histidine tag. To make the plasmid construct, the DNA sequence encoding gerbil pro-IL-18 was amplified by PCR using the primers "jIL18 *Bam*HI sense" 5'-CTA *GGA TCC* GCT GCC ACA CCA GAA GAA GGC-3' and "jIL-18 *Not*I antisense" 5'-GAT *GCGGCCGC* TTA ACT TTG ATG TAA GGG TG-3', and *Pwo* DNA polymerase (Boehringer Mannheim, Laval, Canada), following the manufacturer's instructions. The product was digested with *Bam*HI and *Not*I, and inserted into the bacterial expression vector pQE-30 (Qiagen, Toronto, Canada) previously digested with the same enzymes. The resulting plasmid, pQE30jIL18, was used to transform *E. coli* TOP10F'.

A culture of TOP10F'pQE30jIL18 was grown for 12 h at 37°C with shaking, in 250 ml LB broth supplemented with 100 μg/ml ampicillin, and the cells were harvested by centrifugation. The cell pellet was resuspended in 20 ml binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole) supplemented with 50 mg lysozyme, incubated at 37°C for 1-2 h and sonicated. The lysate was then ultracentrifuged at 40 000 x *g* for 30 min at 4°C, and the cell pellet was dissolved in binding buffer containing 8 M urea. After an overnight incubation at room temperature, the extract was sonicated, ultracentrifuged as above, and the supernatant applied to a Ni-NTA Superflow column (Qiagen) equilibrated with the urea-containing binding buffer. The column was washed with wash buffer made of 20 mM Tris-HCl, pH 7.9, 8 M urea, 0.5 M NaCl, 0.5% Tween-20 and 60 mM imidazole, and the bound proteins were eluted with elution buffer containing 20 mM Tris-HCl, pH 7.9, 8 M urea, 0.5 M NaCl, and 0.5 M imidazole. The eluate was dialyzed against distilled water, lyophilized, and the remaining proteins dissolved in 1 ml phosphate-buffered saline. Protein concentration in the sample was determined with the BCA protein assay kit (Pierce, Rockford, IL), using bovine serum albumin (Sigma) as standard.

Balb/c mice (Charles River) were injected subcutaneously three times, two weeks apart, with 10 μg of the purified recombinant gerbil IL-18 emulsified in complete (first injection) or incomplete (second and third injections) Freund's adjuvant (ICN, Montreal, Canada). The animals were bled 10 days after the last injection and their sera were collected and pooled.

Construction of gerbil IL-18 and caspase-1 vectors for mammalian expression

DNA encoding the full gerbil pro-IL-18 sequence was amplified by PCR from cDNA derived from LPS-stimulated gerbil resident peritoneal cells, using the primers "jIL18 *Eco*RI sense": 5'-CTA *GAA TTC* TCT TGG CCC AGG AAT AAT G-3' and "jIL18 *Not*I antisense" described above, and *Pwo* DNA polymerase (Boehringer Mannheim), according to the manufacturer's instructions. The product was digested with *Eco*RI and *Not*I, and ligated into pCI-neo (Promega), which had been pre-treated with the same enzymes, to produce pjproIL18. Similarly, the coding sequence for gerbil caspase-1 was amplified using the primers "mICE_5' sense" (see above) and "jICE *Xho*I antisense": 5'-GCA *CTC GAG* TTTA ATG TCC TGG GAA GAG G-3', and the product was digested with *Xho*I and ligated into *Pvu*II/*Xho*I-treated pCEP4 (Invitrogen, Carlsbad, CA), to give pjICE. The inserts in pjproIL18 and pjICE were sequenced to verify that no mistakes were introduced during the PCR procedures.

Processing of gerbil pro-IL-18 by caspase-1

COS-7 cells were plated at a density of 4 x 10^5/ml in 60-mm culture dishes (Sarstedt, Newton, NC) in DMEM (GIBCO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 24 mM HEPES and 10% FBS, and incubated overnight at 37°C in a humidified CO_2 atmosphere. They were then transfected with 1 μg of CsCl gradient centrifugation-purified pjproIL18 or pjICE. LipofectAmine reagent (GIBCO) in serum-free, antibiotic-free DMEM was used as transfection vehicle. After a 5-h incubation period in the presence of the DNA-liposome complexes, the medium was removed and replaced with DMEM containing antibiotics but no serum, and the cultures

were incubated for another 72 h. Each dish of transfected cells was scraped with a rubber policeman, the suspensions were centrifuged and the pellets resuspended in 150 µl 10 mM Tris, pH 8.0, containing 5 mM dithiothreitol (DTT). The cells were lysed by 6-7 freeze-thaw cycles, microcentrifuged at 14 000 rpm for 10 min at 4°C, and the supernatants incubated for 30 min at 37°C.

The cell lysate containing gerbil caspase-1 was diluted 1:2, 1:5, 1:10 and 1:25 in Tris-DTT buffer, and 5 µl of each dilution was mixed with 10 µl of the pro-IL-18-containing lysate. The samples were incubated at 37°C for 30 min to allow for the conversion of pro-IL-18 to mature IL-18 by caspase-1. For analysis, the samples were then mixed with 5 µl 4X reducing SDS-PAGE sample buffer (39), boiled, separated by electrophoresis in a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was probed with a 1:500 dilution of the mouse anti-gerbil IL-18 antiserum, washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse Ig antibody. Peroxidase activity on the membrane was visualized using the enhanced chemiluminescence system (ECL; Amersham).

Proliferation-based IL-18 bioassay

COS-7 cells (30 dishes) were transfected as described above with pjproIL18, and incubated at 37°C in a humidified CO_2 atmosphere for 48 h. Half of the dishes were then transfected with pjICE, and all the cultures were incubated for another 48 h. The transfectants were then harvested by scraping the dishes and centrifuging the resulting suspensions, and they were pooled within each group. The cells were resuspended in 2.5

ml Tris-DTT and lysed by repeated freeze-thaw cycles. The lysates were cleared by centrifugation and their soluble fraction incubated at 37°C for 30 min.

Pro-IL18 (from the cells transfected with pjproIL18 alone) and mature IL-18 (from those transfected with pjproIL18 and pjICE) were partially purified from the cell lysates by gel filtration, using Sephadex G-75 columns (Amersham) equilibrated with 10 mM Tris, pH 8.0. Elution fractions of 1.5 ml were collected, and those containing pro- or mature IL-18 were identified by immunoblot and pooled.

The spleen from a 50-60 days old female gerbil or a 42 days old BALB/c mouse (Charles River) were homogenized and the erythrocytes were lysed by osmotic shock using Gey's salts (38). The splenocytes were resuspended at a density of 3×10^6/ml in cRPMI containing 0.5 μg/ml ConA (Sigma), and the suspensions (100 μl/well) were distributed in flat-bottomed 96-well culture plates (Falcon; Becton Dickinson, Lincoln Park, NJ). The pro- and mature IL-18 protein preparations were diluted 1:10 in cRPMI, and 1:2 serial dilutions were made from these. The dilutions were added to the cells (100 μl/well) in triplicates, and the cultures were incubated for 48 h. Each well then received [^{3}H]thymidine (ICN; 1 μCi/well in 20 μl cRPMI) and the cultures were incubated overnight before being harvested onto glass fiber filters, using an automated cell harvester (Skatron, Lier, Norway). The radioactivity incorporated in the cells was finally measured by scintillation counting (LKB Wallac; Pharmacia).

RESULTS

Cloning of gerbil IL-18 and caspase-1

Using the cross-species RT-PCR, 5'RACE and 3'RACE techniques, we have determined the cDNA sequences for gerbil IL-18 and caspase-1 (Fig. 1A and B). The gerbil IL-18 sequence (Fig. 1A) has a 334-bp long 5'UTR followed by a 588-bp open reading frame (ORF), and a short portion (19 bp) of the 3'UTR. The ORF, which is 87% identical to its mouse counterpart (Fig. 2A), encodes an IL-18 precursor protein (pro-IL-18) with a predicted molecular weight (MW) of 22.2 kDa. The protein includes a putative amino-terminal portion that is cleaved off at position Asp^{37} (based on homology with IL-18 sequences from other species) to release the 158-aa mature IL-18. Gerbil mature IL-18 has a predicted MW of 18.0 kDa, and is 90% similar to the mouse counterpart. Its 2 cysteine residues are conserved in the mouse protein; however, mouse mature IL-18 has a third one which is substituted for by a serine residue in the gerbil molecule.

The 1310-bp gerbil caspase-1 sequence and its predicted translation product are shown in Figure 1B. The DNA sequence starts with the initiation codon and includes a 1209-bp ORF, as well as a 3'UTR that extends to the polyA tail. The ORF encodes a precursor protein (caspase-1 precursor) of 402 aa with a predicted MW of 45.3 kDa. The 3 proteolytic cleavage sites (Asp^{122}, Asp^{296} and Asp^{314}) required to generate the p20 (Gly^{123} - Asp^{296}) and p10 (Gly^{315} - Asp^{402}) subunits of the mature heterodimeric protein out of the precursor are all present, based on the mouse caspase-1 sequence (Fig. 2B). The gerbil protein contains 11 cysteine residues, 10 of which are conserved in mouse caspase-1, including the active cysteine residue of the protease situated in the p20 subunit.

A

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                                20                  40                  60                  80
                    ATTGAGTGAAAACAATGCTGTGGTTGGTAAACCAAGCCTTCTTTCACTTCTCCCCTGTGGAGTACTGCCTGCCTCACACAGAGAGAAGCTTC

  100                 120                 140                 160                 180                 200
GGAAGCCTGCCATAATCTTCAGGAGCCAGGCACAGCAAGCAGTCCAACTGCAGACTGGCCTTTTCTCTGACTGCAGCTGGATCCACACCCGGGCTGCAGATTCTCTGCGCCTCCATGCCC

  220                 240                 260                 280                 300                 320
TCTGTATTCCTGGCTGCCTGCTCTGGCCCCTGAGAGGACTGCCAATTCTGCAACCTCCAGTGTCAGGAAGAGGAAAGAGGCCGCCAAGCTTCCAGAGCACTTCCTCTTGGCCCAGGAATA

  340                 360                 380                 400                 420                 440
ATGATGGCTGCCACACCAGAAGAAGGCTCTTGCATCAACTTCAAGGAAATAATGTTTATTGACAACACACTTTACTTTATACCTGAAGATAATGGAGACCTGGAATCAGACAGCTTTGGC
 M  M  A  A  T  P  E  E  G  S  C  I  N  F  K  E  I  M  F  I  D  N  T  L  Y  F  I  P  E  D  N  G  D  L  E  S  D  S  F  G
  460                 480                 500                 520                 540                 560
AGGCTTAGCTCTACAGCCACGGTGATACGAAATATAAATGAGCAAGTTCTCTTCGTTGACAGAGGAAAACAGCCAGTGTTTGAGGACATGACTGATGCGGAACAAGAGGCTAATGGACCC
 R  L  S  S  T  A  T  V  I  R  N  I  N  E  Q  V  L  F  V  D  R  G  K  Q  P  V  F  E  D  M  T  D  A  E  Q  E  A  N  G  P

  580                 600                 620                 640                 660                 680
CAGACCAGACTGATAATATATATGTATAAGGATACTGAAGCAAGGGGGCTGGCTGTAACCCTCTCTGTGAAGGATACAACAATGGCTACCCTCTCCTGTAAGAACAAAATCATTTCCTTT
 Q  T  R  L  I  I  Y  M  Y  K  D  T  E  A  R  G  L  A  V  T  L  S  V  K  D  T  T  M  A  T  L  S  C  K  N  K  I  I  S  F
  700                 720                 740                 760                 780                 800
GAGGAAATGAATCCACCTGAAAATATTGATGATACAGAAAGTGATCTCATATTCTTTCAGAAACGTGTTCCAGGACACAATAAGATGCAATTTGAATCTTCACTGTACAAAGGGCACTTT
 E  E  M  N  P  P  E  N  I  D  D  T  E  S  D  L  I  F  F  Q  K  R  V  P  G  H  N  K  M  Q  F  E  S  S  L  Y  K  G  H  F

  820                 840                 860                 880                 900                 920
CTAGCTTGCCAAAAGGAGGAAGACGCTTTCAAACTCATCTTGAAAAAAAAGGATGAAAATGGGGATAAATCTGTAATGTTCACTGTTACACCCTTACATCAAAGTTAAGCATTAAAGTTT
 L  A  C  Q  K  E  E  D  A  F  K  L  I  L  K  K  K  D  E  N  G  D  K  S  V  M  F  T  V  T  P  L  H  Q  S  *

  940
TTGTGTT
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B

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                  20                  40                  60                  80                 100                 120
ATGGCTGATGCCACCCTGAGGTCAAAGAGGACAAAATTTATCAATTCAGTGGATGGAGGGATAATAAATGGTTTGCTGATGAACTTTTAGAGAAGAGAGTGCTGAACGAGGAAGAGATG
 M  A  D  A  T  L  R  S  K  R  T  K  F  I  N  S  V  D  G  G  I  I  N  G  L  L  D  E  L  L  E  K  R  V  L  N  E  E  E  M
                 140                 160                 180                 200                 220                 240
GAGAGAATAAAAGCTGAAAATGCCACTGTTAAGGACAAGGCAAGACACCTGTGTGACTCCGTCACTCAAAAAGGGCCTGAGGCAAGCCAAATCTTGATCACTTACATTTGTAAAGAAGCC
 E  R  I  K  A  E  N  A  T  V  K  D  K  A  R  H  L  C  D  S  V  T  Q  K  G  P  E  A  S  Q  I  L  I  T  Y  I  C  K  E  A

                 260                 280                 300                 320                 340                 360
TGCTTTGTGGCAAAAAAGATGGAGCTTGAATCAGGTCCACCAGCTGAAAATGTTGCTACTAAAGAAGATTTCAAGGGAAGAAGTCTTTTCTCCTCAGAAACAAAGCAGACCCAGAACAAA
 C  P  V  A  K  K  M  E  L  E  S  G  P  P  A  E  N  V  A  T  K  E  D  F  K  G  R  S  L  F  S  S  E  T  K  Q  T  Q  N  K
                 380                 400                 420                 440                 460                 480
GAAGATGGCGCATGCCCAGGACCAAGTGGGAGCCTCAAGATTTGTCCCTTAGAAACAGCCCAAAAGATTTGGAATGAAAATCCTTCAGAGATTTATACAATAATGGATAAATCCACTCGT
 E  D  G  A  C  P  G  P  S  G  S  L  K  I  C  P  L  E  T  A  Q  K  I  W  N  E  N  P  S  E  I  Y  T  I  M  D  K  S  T  R
                 500                 520                 540                 560                 580                 600
ACACGTCTTGCCCTCATTATCTGCAACACAGTGTTTGAACATCTTCCTAGACGGGATGGAGCCGATGTTGACCTCAGAGAAATGAAGTTGCTGCTGCAGAATCTGGGATATACTGTGAGA
 T  R  L  A  L  I  I  C  N  T  V  F  E  H  L  P  R  R  D  G  A  D  V  D  L  R  E  M  K  L  L  L  Q  N  L  G  Y  T  V  R
                 620                 640                 660                 680                 700                 720
GTGAAAGAAAACCTCACAGCTGCGGAGATGAAGGAAGAGGTGAAAGAGTTTGCTGCCCAGCCCGAACACAAGACTTCTGACAGTGCTTTCCTTGTTTTCATGTCTCATGGAATACTGGAG
 V  K  E  N  L  T  A  A  E  M  K  E  E  V  K  E  F  A  A  Q  P  E  H  K  T  S  D  S  A  F  L  V  F  M  S  H  G  I  L  E
                 740                 760                 780                 800                 820                 840
GGAGTATGTGGGAAAACACACTCTGATGAAGCTGCAGATGTTTTCAAAGTTGGCACAATCTTTGAAATGATGAACACTTTGAACTGCCCAAGCTTGAAAGACAAGCCTAAGGTTATCATC
 G  V  C  G  K  T  H  S  D  E  A  A  D  V  F  K  V  G  T  I  F  E  M  M  N  T  L  N  C  P  S  L  K  D  K  P  K  V  I  I
                 860                 880                 900                 920                 940                 960
ATTCAGGCCTGCCGGGGAGAGAGAAAAGGAGTGGTGTTCGTAAAAGATTCAGCAGGTAAATCTGAAAAGGAGCTCTCAACGGATGCAAATTTTGAAGATGACGGCATTAAGAAGGCCCAT
 I  Q  A  C  R  G  E  R  K  G  V  V  F  V  K  D  S  A  G  K  S  E  K  E  L  S  T  D  A  N  F  E  D  D  G  I  K  K  A  H
                 980                1000                1020                1040                1060                1080
ATAGAGAAGGATTTTATTGCTTTCTGCTCTTCAACACCAGATAATGTCTCTTGGAGACATCCTGTCCGAGGCTCTGTTTTCATTGAGAAACTCATCAAACTTATGAAAGAGTATGCCTGG
 I  E  K  D  F  I  A  F  C  S  S  T  P  D  N  V  S  W  R  H  P  V  R  G  S  V  F  I  E  K  L  I  K  L  M  K  E  Y  A  W

                1100                1120                1140                1160                1180                1200
TCTTGTGACTTGGAGGACATATTCAGAAAGGTTCGATTCTCATTTGAGCAGCCGGATTCGATGGTCCAGATGCCCACCAGTGAAAGAGTGACCTTGACAAAACGTTTCTACCTCTTCCCA
 S  C  D  L  E  D  I  F  R  K  V  R  F  S  F  E  Q  P  D  S  M  V  Q  M  P  T  S  E  R  V  T  L  T  K  R  F  Y  L  F  P
                1220                1240                1260                1280                1300
GGACATTAAATAAGAATCCAGGAGACCCATCCTTCTGTACCTGTTTTGGGAATCATCTCAATTAGAAGCTAATATGTCTCTAAATGATTCAATATGTCTAAAAAAAAAAAAAAAAA
 G  H  *
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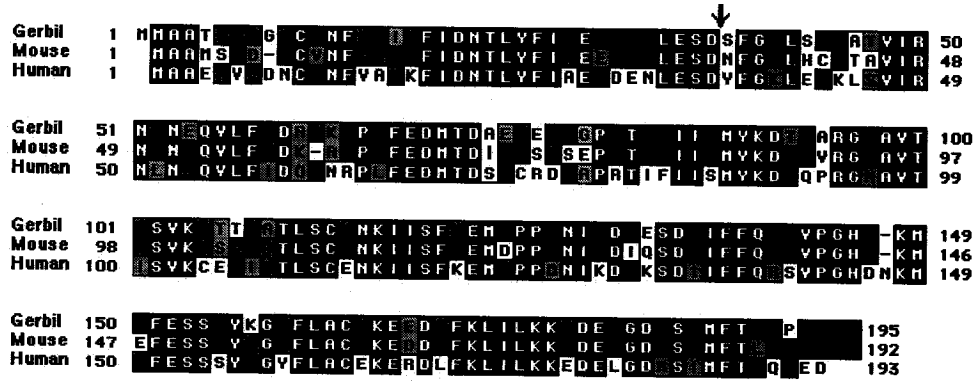
Figure 1. Nucleotide sequences and corresponding amino acid sequences of gerbil IL-18 (A) and gerbil caspase-1 (B) cDNAs. The nucleotide positions are numbered above the DNA sequences, and asterisks identify the stop codons. The GenBank accession numbers for gerbil IL-18 and gerbil caspase-1 are AY095932 and AF390032, respectively.

A



B

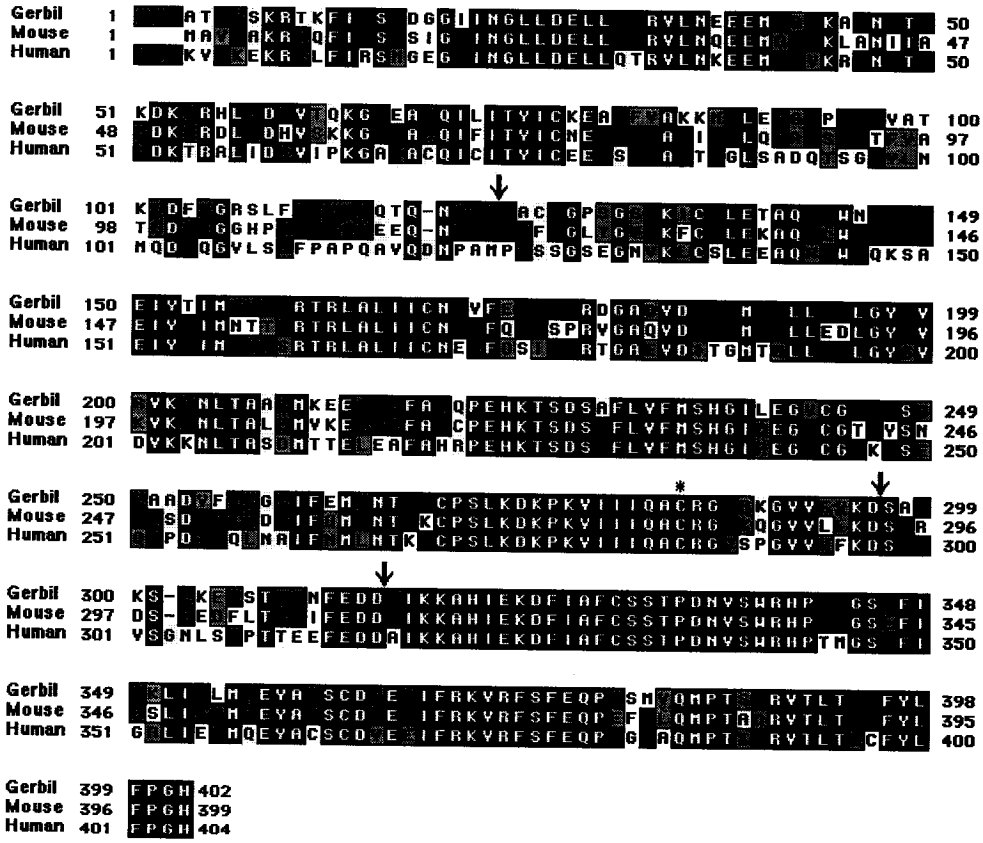


Figure 2. Alignment of the deduced amino acid sequences of gerbil, mouse (GenBank Accession number D49949) and human (D49950) pro IL-18 (A), and gerbil, mouse (L28095) and human (M87507) precursor caspase-1 (B). The amino acid residues are numbered at the beginning and the end of each line of the alignments. Conserved residues are boxed in black, identical residues in dark grey and similar residues in light grey. The arrows indicate the proteolytic sites recognized by caspase-1. The asterisk identifies the catalytic cysteine residue of caspase-1.

Overall, gerbil and mouse caspase-1 precursor proteins are 85% identical at the nucleotide level and 83% similar at the aa level.

Convertase activity of gerbil caspase-1 on pro-IL-18

The cDNA sequences encoding gerbil pro-IL-18 and caspase-1 precursor were inserted in mammalian expression vectors, and the constructs were used to transfect COS-7 cells. Lysates from the cells expressing caspase-1 were incubated with those containing pro-IL-18 and the conversion of pro-IL-18 to mature IL-18 by caspase-1 was analyzed by western blot. As shown in Figure 3, processing of pro-IL-18 occurred in a dose-dependent manner.

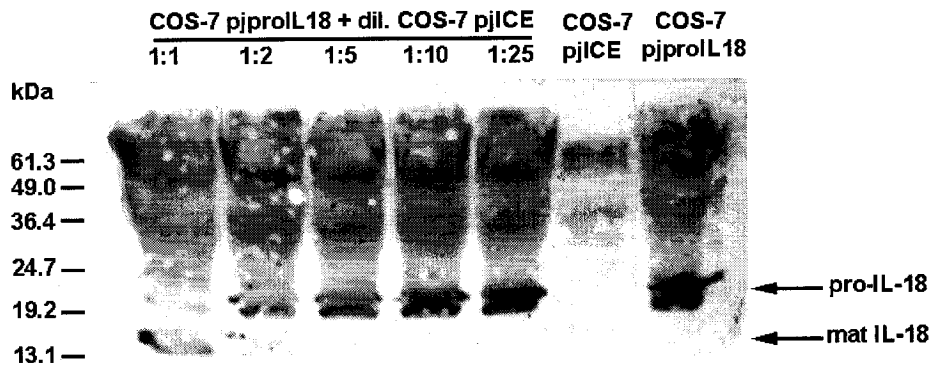


Figure 3. Conversion of gerbil pro IL-18 to mature IL-18 by gerbil caspase-1. Lysates from COS-7 cells containing pro IL-18 or activated caspase-1 were mixed together, incubated at 37°C for 30 minutes, separated in a 15% SDS-polyacrylamide gel under reducing conditions, and analyzed by immunoblot using antiserum raised against recombinant gerbil IL-18 (1:500 dilution).

Bioactivity of mature gerbil IL-18

Pro-IL-18 and mature IL-18 were partially purified from lysates from COS-7 cells transfected with pjproIL18 alone or subsequently with pjICE, respectively. To determine whether the molecules were bioactive, different dilutions of the preparations were incubated with gerbil or mouse spleen cells and the proliferation of these cells was measured. The preparation containing mature IL-18 induced the proliferation of gerbil (Fig. 4A) and mouse (Fig. 4B) splenocytes in a dose-dependent manner. The pro-IL-18 preparation did not have any appreciable effect on the spleen cells.

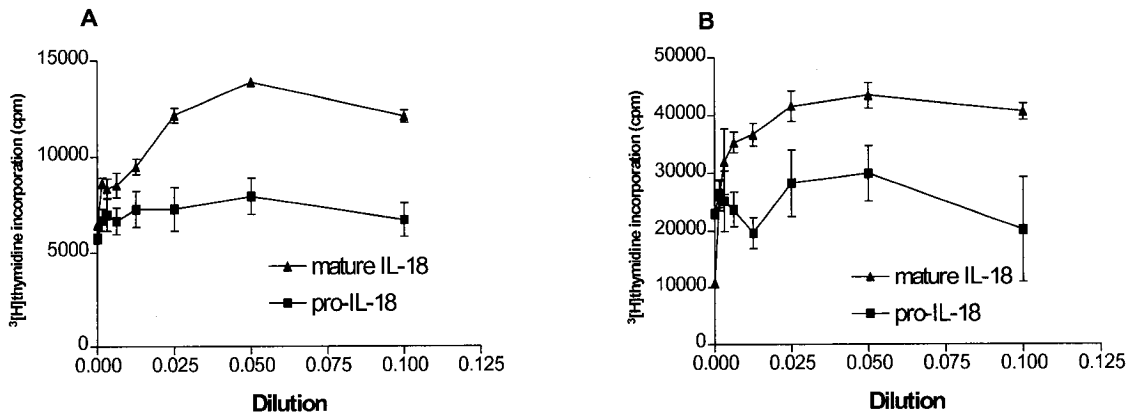


Figure 4. Bioactivity of gerbil IL-18. Gerbil (A) or mouse (B) spleen cells were incubated with different dilutions of partially purified preparations of mature (▲) or pro (■) IL-18. Proliferation of the splenocytes in response to the stimuli was measured by [^{3}H]thymidine incorporation. Results are expressed as mean cpm of triplicate cultures ± SE.

DISCUSSION

The cDNAs encoding gerbil IL-18 and caspase-1 were identified by RT-PCR, using primers based on known, homologous sequences from other species, and by 3'/5'RACE. Gerbil pro-IL-18 coding sequence was found to have a nucleotide identity of 87% with its mouse counterpart and the aa sequence of gerbil mature IL-18 had a 90% similarity with mouse mature IL-18. This high homology allows the gerbil protein to be active on mouse lymphocytes. Caspase-1 precursor had an 85% nucleotide identity and an 83% aa similarity to its murine counterpart. All the proteolytic sites required to derive the p10 and p20 subunits for the precursor molecule are conserved between the two species. Likewise, the catalytic cysteine residue present in p20 is also conserved (Cys^{281} in the mouse, Cys^{285} in the gerbil). Alternative splicing of the human caspase-1 mRNA gives rise to 5 isoforms of caspase-1, each with different apoptotic activities (40). If the same phenomenon occurs with gerbil caspase-1, the sequence we are reporting would correspond to the α isotype, encoded by the full length mRNA.

Co-transfection of COS-7 cells with plasmids encoding human caspase-1 and IL-1β precursor led to the expression of active caspase-1 and secretion of mature IL-1β in the culture supernatants (28). Similarly, co-expression of porcine caspase-1 and pro-IL-18 in insect cells using a baculovirus system resulted in secretion of mature IL-18 by the cells (41). In addition, overexpression of human or mouse caspase-1 in mammalian or insect cells caused the cells to undergo apoptosis (28, 40, 42). While COS-7 cells co-transfected with pjICE and pjproIL18 overexpressed both proteins in their precursor form (data not shown), they did not, however, appear to produce active caspase-1; there was no

significant difference in viability between these cells and cells transfected with pCI-neo, and no mature IL-18 could be detected in the culture supernatants or in the cell lysate. In our system, the only way to obtain active gerbil caspase-1 was to lyse the cells containing caspase-1 precursor in a DTT-containing buffer and incubate the lysate at 37°C for 30 min. Caspase-1 activated this way was able to convert pro-IL-18 into a mature IL-18 that showed bioactivity on gerbil and mouse spleen cells.

The cloning and characterization of gerbil IL-18 and caspase-1 now enables us to produce the recombinant molecules in a purified form. In addition, other immunological reagents, such as polyclonal or monoclonal antibodies against the proteins or nucleic acid probes based on their cDNA sequences, can also be generated and used to study the role of these molecules *in vivo* or *in vitro*, with regards to various pathological conditions.

ACKNOWLEDGEMENTS

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CONNECTING STATEMENT III

The cloning, expression and characterization of the gerbil Th1-enhancing cytokines interleukin (IL)-12 and IL-18 were reported in Manuscripts II and III, respectively. These cytokines, when used as genetic adjuvants co-injected with a DNA vaccine, should enhance the immune response to the antigen encoded by the vaccine and ensure that this response is of the Th1 type. Another cytokine that can act as an adjuvant for DNA vaccination is granulocyte/macrophage colony-stimulating factor (GM-CSF). This hematopoietic factor acts on bone marrow progenitor cells to make them differentiate into granulocytes, macrophages and dendritic cells, the latter two being important professional antigen presenting cells (APCs). It enhances the killing capacity and the antigen-presentation ability of macrophages and is involved in the development of dendritic cells, which are central in the induction of primary immune responses. An increased number of circulating activated APCs could be helpful in the context of DNA vaccination, especially if the *in vivo* level of antigen expression is low. We were interested to try this genetic adjuvant in gerbils, together with our DNA vaccine. Manuscript IV thus reports the cloning and characterization of gerbil GM-CSF.

MANUSCRIPT IV

Molecular cloning and expression of gerbil granulocyte/macrophage colony-stimulating factor*

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ABSTRACT

Using a combination of cross species reverse transcriptase-polymerase chain reaction (RT-PCR) and 3' rapid amplification of cDNA ends (RACE) techniques, we cloned the cDNA encoding gerbil granulocyte/macrophage colony-stimulating factor (GM-CSF). The open reading frame had 81% nucleotide identity with its mouse counterpart, while the mature protein had 80% similarity with mature mouse GM-CSF. COS-7 cells transfected with gerbil GM-CSF cDNA secreted high levels of bioactive GM-CSF, as their supernatant stimulated gerbil bone-marrow cell proliferation and colony formation in semi-solid medium.

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*) is permissive to several infections, and is an exciting animal model alternative for the study of diseases that cannot infect mice and rats. It is currently being used in studies on infectious diseases of viral (1), bacterial (2, 3) and parasitic (4-6) origins. However, the lack of commercially available gerbil-specific immunological reagents has hampered immunopathological studies on those diseases. Cross-species bioassays can be used for a few well-conserved cytokines (IL-2, IL-4, TNF-α) (7), but most cytokines are species-specific and inactive on cells from other species. Furthermore, *in vivo* injection of cytokines from other species is not advisable since the host could eventually raise an immune response against the foreign molecule, which would abrogate its effect. Thus, there is a need to identify gerbil cytokine coding sequences and use them to produce immunological reagents.

GM-CSF is a hematopoietic cytokine produced by several cell types including T cells, macrophages, mast cells, endothelial cells, epithelial cells and fibroblasts. Its main activity is to support the production of granulocytes (mainly neutrophils and eosinophils) and macrophages from bone marrow progenitor cells (8). Therefore, GM-CSF is an obvious molecule to be used clinically to counteract the effects of treatments that deplete those cell types (9). GM-CSF is being used, among other things, to treat neutropenia in cancer patients undergoing chemotherapy (10), in AIDS patients during therapy (11), and is administered after bone marrow transplantation (12).

GM-CSF also activates mature eosinophils and neutrophils, enhances their survival (13), and increases their ability to kill infectious agents (14). Furthermore, it enhances the killing capacity of macrophages (15), as well as their antigen-presentation ability (16) by increasing their expression of MHC class-II molecules, adhesion molecules and costimulatory factors. GM-CSF is also involved in the development (17) and activation (18) of dendritic cells, which are central in the induction of primary immune responses.

Because of its differentiation and activation properties on antigen-presenting cells as well as some other immunomodulatory effects, GM-CSF is an attractive molecule to be used as an adjuvant in vaccination strategies, and several studies have already been published in this field (19). The cytokine, either injected as a soluble protein, encapsulated in liposomes, or as DNA (GM-CSF-encoding sequence), generally improved the immune response to the co-administered antigen, protection against disease, and this with virtually no side-effects. Herein, we report the cloning, expression and characterization of gerbil GM-CSF.

MATERIALS AND METHODS

Collection of peritoneal cells and isolation of RNA

Resident peritoneal cells from 50-60 days old female gerbils (Charles River. St. Constant, Canada) were harvested by lavage of the peritoneal cavity as described (20), with 10 ml of ice-cold 10-RPMI, made of RPMI 1640 medium (GIBCO, Burlington,

Canada) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 24 mM HEPES (Sigma, St. Louis, MO) and 10% FBS (GIBCO). The erythrocytes were lysed with Gey's salts (20), and the remaining cells were resuspended in the above medium at a density of 5 x 10^6/ml. The suspension was aliquoted in a 24-well plate at 1.5 ml/well, and the cells were incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere, with 1 μg/ml LPS (*Escherichia coli* 0111:B4; Sigma). The stimulated cells were harvested and their RNA isolated with TRIzol reagent (GIBCO), according to the manufacturer's instructions.

cDNA was generated from 2 μg total RNA in a 50-μl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 4 mM dithiothreitol, 1 mM dNTP (GIBCO), 8 U of RNasin ribonuclease inhibitor (Promega Corp., Madison, WI), 100 U of Moloney MuLV reverse transcriptase (GIBCO) and 0.2 μg oligo-dT primer (GIBCO) for regular PCR, or 20 pmol anchor primer 5'-GGC CAC GCG TCG ACT AGT $AC(T)_{17}$-3' for 3'RACE. The reaction was incubated at 37°C for 1 hour, and the enzyme was then inactivated at 95°C for 5 minutes. All subsequent PCRs contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 - 3.5 mM $MgCl_2$, 40 μM dNTP, 50 pmol of both sense and antisense primers, 2.5 U *Taq* DNA polymerase and 5 μl of cDNA, in a total volume of 50 μl. The PCR conditions always consisted of: 30 s at 95°C, 1 min at 55°C, and 2 min at 72°C for 35 cycles, and a final cycle with the extension step at 72°C for 8 min. PCR products were separated by electrophoresis in 1-2% agarose gels containing ethidium bromide. All relevant DNA bands were sliced out, purified using the Sephaglas BandPrep Kit (Amersham Pharmacia Biotech, Baie d'Urfé, Canada), inserted in the cloning vector pGEM-T Easy (Promega), and sequenced.

Cloning of gerbil GM-CSF

Cloning of gerbil GM-CSF cDNA was done in two steps. First, PCR was done with the primers "GM-CSF_5' sense": 5'-AAG GCT AAG GTC CTG AGG AGG-3', which was based on the 5' untranslated region of the mouse GM-CSF cDNA (GenBank accession number X03019), and "GM-CSFint antisense": 5'-CTT CAG GCG GGT CTG CAC AC-3', also based on the mouse sequence. The 255 bp product was cloned, and its sequence used to design the primer "jGM-CSF sense2": 5'-TCA AAG AAG CTC TGA GCC-3'. This primer was then used in 3'RACE together with the adapter primer 5'-GGC CAC GCG TCG ACT AGT AC-3'. The reaction gave a product of 503 bp, which was cloned and sequenced, and found to contain the rest of the coding sequence and the complete 3'UTR, up to the poly(A) tail.

Expression of gerbil GM-CSF in COS-7 cells

DNA encoding the full gerbil GM-CSF protein was amplified by PCR from cDNA derived from LPS-stimulated peritoneal cells, using the primers "jGM-CSF*Xho*I sense": 5'-GCA CTC GAG GTC CTG AGG AGG ATG TG-3' and "jGM-CSF*Sal*I antisense": 5'-TCA GTC GAC TCA CTC TTG GAC TGG CTC-3' and *Pwo* DNA polymerase (Boehringer Mannheim, Laval, Canada), according to the manufacturer's instructions. The product was digested and ligated into *Xho*I/*Sal*I-treated pCI-neo (Promega), to yield the plasmid pjGM-CSF. The insert in this plasmid was sequenced to verify that no mistakes were introduced during the PCR procedure.

COS-7 cells were plated at a density of 4 x 10^5/ml in 60-mm culture dishes (Sarstedt, Newton, NC) in DMEM (GIBCO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 24 mM HEPES and 10% FBS, and incubated overnight. They were then transfected with 1 μg of CsCl gradient centrifugation-purified pjGM-CSF or pCI-neo (control vector), using LipofectAmine reagent (GIBCO) in serum-free, antibiotic-free DMEM. After a 5-hour incubation period in the presence of the DNA-liposome complexes, the medium was removed and replaced with DMEM containing antibiotics but no serum. The cultures were incubated for another 72 hours, and the cells and their supernatants were harvested and kept at 4°C.

Bone marrow cell proliferation assay

Tibias and femurs from a gerbil were flushed with cold RPMI supplemented with 2% FBS, and the bone marrow was collected in a 50 ml centrifuge tube. The clumps were separated by vigorous pipetting until a single-cell suspension was obtained. The red blood cells were lysed with Gey's salts, and the remaining cells were resuspended at a density of $5x10^5$ /ml in 15-RPMI (RPMI + 15% FBS) and 0.1 ng/ml LPS. The cells were distributed in a 96-well culture plate (100 μl/well), and an equal volume of 1:10 serially diluted COS-7 supernatant containing gerbil GM-CSF was added to the cells. Control wells received 1:10 serially diluted supernatant from COS-7 cells transfected with the empty vector pCI-neo for 72 hours. Each condition was done in triplicates. The cells were incubated for 4 days at 37°C in a humidified 5% CO_2 atmosphere, and 1 μCi [^{3}H]thymidine in 15-RPMI was added to each well in a volume of 25 μl. The cultures were incubated for another 24 hours and harvested onto glass fiber filters, using an

automated cell harvester (Skatron, Lier, Norway). The radioactivity incorporated in the cells was finally measured by scintillation counting (LKB Wallac; Pharmacia).

Colony formation assay

Bone marrow cells were obtained as described above, and added to a density of 7.5 x10^4/ml to warm 15-RPMI (42°C) containing 0.1 ng/ml LPS and 0.4% Bacto Agar (Difco, Detroit, MI). One milliliter aliquots were then quickly distributed in 6-well plates already containing 150 µl of undiluted COS-7 supernatant containing gerbil GM-CSF or control supernatant. The medium was left to solidify at room temperature for 15 minutes, and the cultures were then incubated at 37°C in a well humidified, 5% CO_2 atmosphere, for 10 days.

RESULTS

Nucleotide and amino acid sequences of gerbil GM-CSF

A 590 bp cDNA sequence for gerbil GM-CSF was determined (Fig. 1), which starts with the initiation codon, and includes a 438 bp ORF, as well as a complete 3' UTR containing a poly-adenylation signal sequence AAUAAA and a poly(A) tail. The ORF is 81% identical to that of mouse GM-CSF, and encodes a 146 aa precursor polypeptide with a N-terminal 17 aa secretion signal peptide, based on the mouse (21) and human (22) GM-CSF.

```
        10                  30                  50
ATGTGGCTGCAGAATTTACTTTTCCTGAGCATTGTGGTCTACAGCTTCTCAGCACCCACC
 M  W  L  Q  N  L  L  F  L  S  I  V  V  Y  S  F  S  A  P  T

        70                  90                  110
CATTCACCCATCACTGTCACCCAGCCTTGGAAGCATGTAGATGCCATCAAAGAAGCTCTG
 H  S  P  I  T  V  T  Q  P  W  K  H  V  D  A  I  K  E  A  L

        130                 150                 170
AGCCTCCTGGAAAAAATGCTTAAAATCCCTGCCATGCTGGATGAAGACGACGTAGACATT
 S  L  L  E  K  M  L  K  I  P  A  M  L  D  E  D  D  V  D  I

        190                 210                 230
GTCTCTGAAGAATTCTCCGTCCAGAGGCCAACCTGTTTGCAGAAACGCCTGAAGGTATAC
 V  S  E  E  F  S  V  Q  R  P  T  C  L  Q  K  R  L  K  V  Y

        250                 270                 290
GAGCAAGGACTACGGGGCAACTTCACCAGATTCAGGGGCACCTTGGCCATGATAGCCAGA
 E  Q  G  L  R  G  N  F  T  R  F  R  G  T  L  A  M  I  A  R

        310                 330                 350
CACTACCAGAAGTACTGCCCTCCCACCCCGGAAGATGAATGTGAGACAGAAGTCACCACC
 H  Y  Q  K  Y  C  P  P  T  P  E  D  E  C  E  T  E  V  T  T

        370                 390                 410
TTTGGGGATTTCATAGACAGCCTTAAAAACTTTCTGTTTGACATCCCCTTTGACTGCTGG
 F  G  D  F  I  D  S  L  K  N  F  L  F  D  I  P  F  D  C  W

        430                 450                 470
GAGCCAGTCCAAGAGTGAGACAGCCCGGCCAAGATCTTGATCCAACTTCTCATACGGCTG
 E  P  V  Q  E  *

        490                 510                 530
CTTTTGTGCCTATGCAATGAGCCAGCAACTCAGGATTTCTGCCTTGAAGGGATCTAGGGG

        550                 570
ATGTCTTACCATAATAATAAATTATTCAAATGTGAAAAAAAAAAAAAAAA
```

Figure 1. Nucleotide sequence and corresponding amino acid sequence of gerbil GM-CSF cDNA. The nucleotide positions are numbered above the DNA sequences. An asterisk identifies the stop codon and the consensus poly(A) addition sequence is shown in bold characters. The arrow shows the putative signal peptide cleavage site and the potential N-linked glycosylation site is underlined. The GenBank accession number for gerbil GM-CSF is AF387363.

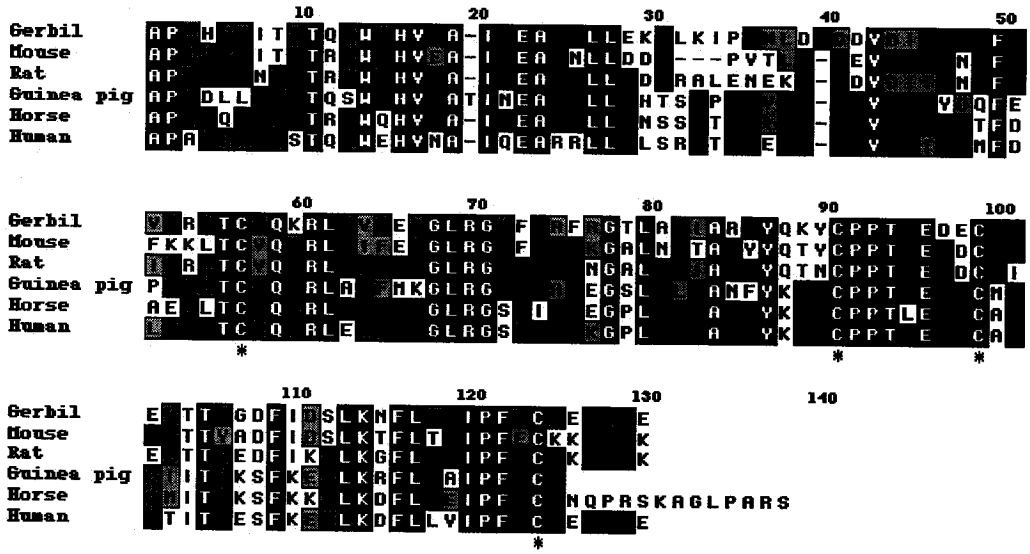


Figure 2. Alignment of the deduced amino acid sequence of mature gerbil, mouse (GenBank Accession number X03019), rat (P48750), horse (AAK72108), guinea pig (Q60481) and human GM-CSF (M10663). The amino acid residues are numbered above the alignments. Conserved residues are boxed in black, identical residues in dark grey and similar residues in light grey. Asterisks identify the cysteine residues.

proteins. The calculated molecular weight of precursor gerbil GM-CSF is 16.9 kDa, while that of the mature cytokine is 14.8 kDa. Mature gerbil GM-CSF (Fig. 2) is 80% similar to its mouse counterpart. The four cysteine residues thought to form two intramolecular disulfide bonds in mouse, human (23) and all other known GM-CSFs are also conserved in the gerbil molecule. While mouse GM-CSF has two potential N-linked glycosylation sites, the gerbil protein only has a single one (Fig. 1).

Expression and analysis of gerbil GM-CSF

COS-7 cells were transfected with the plasmid pjGM-CSF (or pCI-neo as control) for 72 hours, and their supernatants were harvested. To determine whether the conditioned media

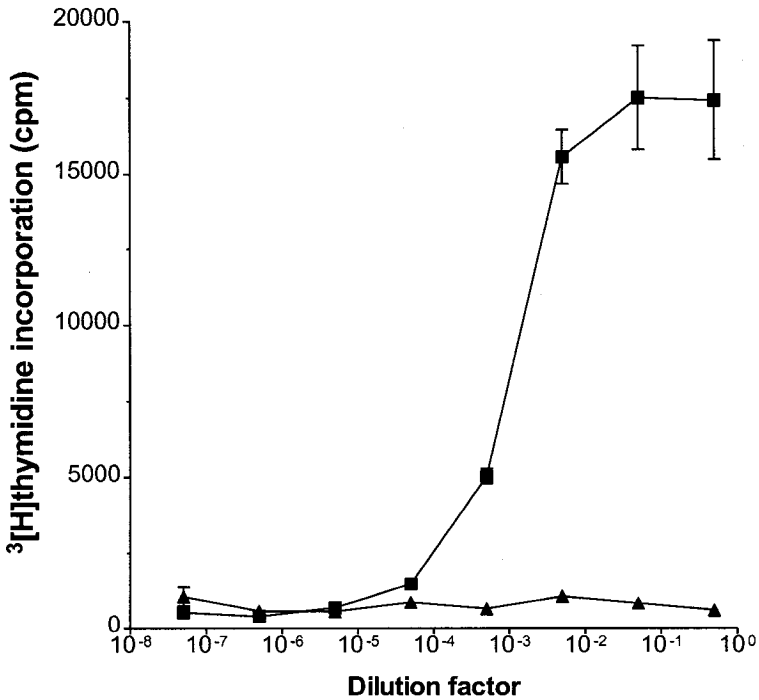


Figure 3. Stimulation of gerbil bone marrow cell proliferation by gerbil GM-CSF. Bone marrow cells were incubated with 1:10 serially diluted supernatants from a 72-h culture of COS-7 cells transfected with pjGM-CSF (■) or with the empty vector pCI-neo (▲). Incorporated [^{3}H] thymidine was then determined as an indication of cell proliferation. Results are expressed as mean cpm of triplicate cultures ± SE.

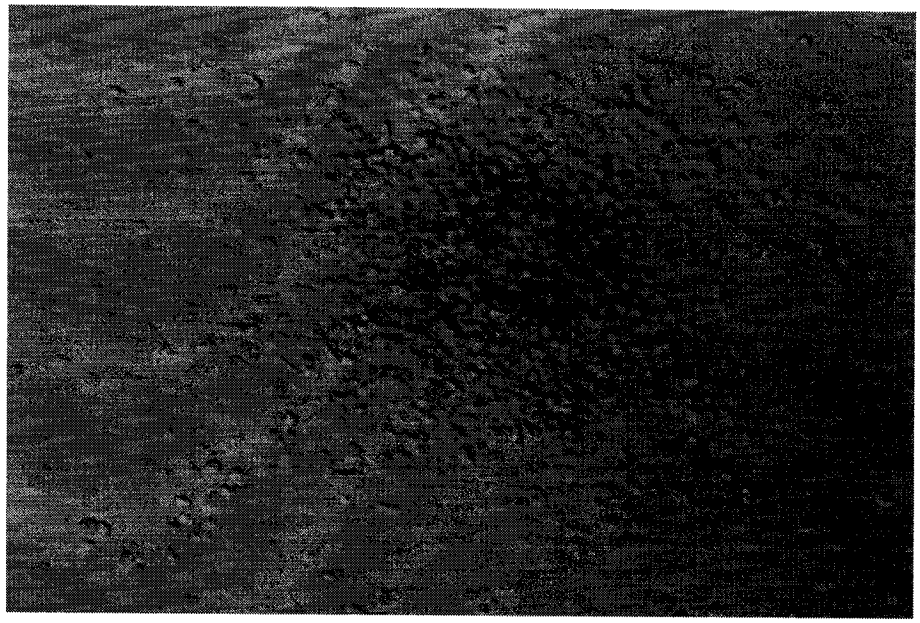
contained GM-CSF that was bioactive, we first performed a bone marrow cell proliferation assay. As shown in Figure 3, the supernatant from the cells transfected with pjGM-CSF induced the proliferation of gerbil bone marrow precursor cells in a dose-dependent manner. The highest level of proliferation was obtained with dilutions of $5x10^{-1}$ and $5x10^{-2}$, and activity was completely titrated out at $5x10^{-6}$. Control supernatants did not stimulate appreciable cell proliferation.

The bioactivity of the supernatants was also tested in a colony-formation assay (Fig. 4). While control supernatants did not stimulate colony formation at all when incubated with gerbil bone marrow precursor cells, culture medium conditioned by COS-7 cells expressing gerbil GM-CSF stimulated the formation of colonies composed of small, tightly grouped cells, as well as bigger cells migrating away from the focus of proliferation.

DISCUSSION

Concurrent experiments being carried out in our laboratory prompted us to clone the cDNA encoding GM-CSF in gerbils. Using cross-species RT-PCR and 3'RACE techniques, we identified a 438-bp ORF that had an 81% identity with that of mouse GM-CSF. Gough and co-workers (24) reported that the mRNA encoding murine GM-CSF contains two functional start codons. They suggested, on the basis of N-terminal amino acid composition, that translation products initiated from the first (5' proximal) AUG

A



B

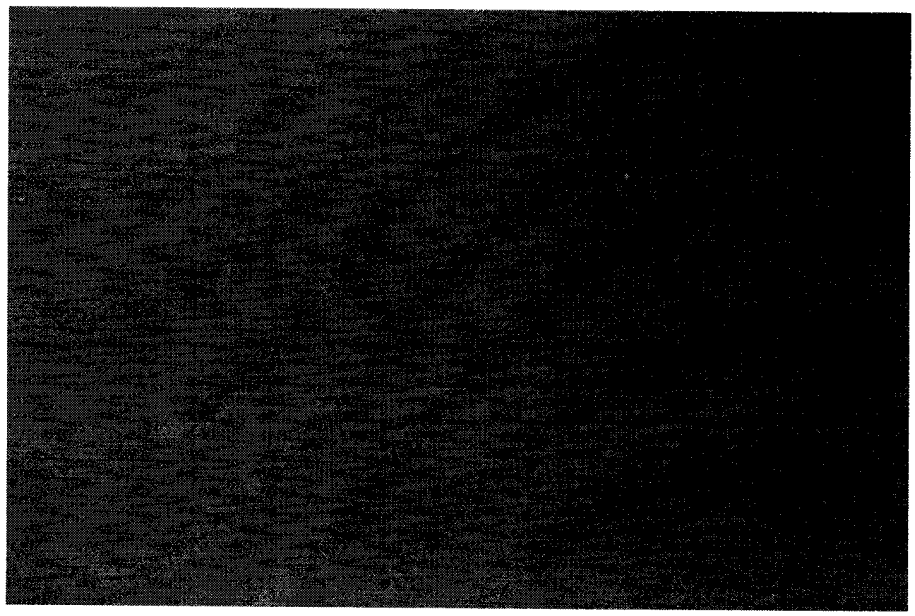


Figure 4. Colony-formation activity of gerbil GM-CSF on gerbil bone marrow cells. Bone marrow cells were incubated with medium containing agar and supernatant from pjGM-CSF- (A) or pCI-neo – (B) transfected COS-7 cells.

could be integral membrane proteins, while those starting from the second AUG are secreted. Since the "GM-CSF_5' sense" primer used to clone the 5' end of the gerbil GM-CSF coding sequence was based on the sequence immediately upstream of the second AUG of the mouse GM-CSF sequence, the gerbil sequence we identified and expressed corresponds to the secreted form of the cytokine.

Mature gerbil GM-CSF protein has a similarity of 80% with its mouse counterpart. Although both molecules differ substantially in the region 27-40, where the gerbil protein has four extra amino acid residues, gerbil GM-CSF is still bioactive on mouse bone marrow cells (data not shown). This would suggest that this region is not critical for binding to the murine receptor and for bioactivity, although it has been previously reported that amino acid residues 24-37 of mouse GM-CSF are critical for function (25). Furthermore, it was also reported that residues Asp^{92}, Thr^{98} and Asp^{102} of mouse GM-CSF are essential for proper interaction of the ligand with its receptor (26). In gerbil GM-CSF, while the last two of those residues are conserved (Thr^{102} and Asp^{106}), the first one is not. However, its corresponding residue, Glu^{96}, is similar in shape and function to Asp, and therefore could act the same way. Alternatively, the preceding residue, Asp^{97}, could be the one interacting with the receptor. COS-7 cells transfected with a plasmid encoding gerbil precursor GM-CSF secreted high levels of bioactive GM-CSF. Supernatants from those cells stimulated cell proliferation from gerbil bone marrow precursors and formation of colonies in semi-solid medium that appeared exactly as previously described GM-CSF- stimulated colonies (27).

The cloning, characterization and expression of gerbil GM-CSF can now allow the generation of gerbil-specific immunological reagents, such as recombinant GM-CSF,

anti-GM-CSF antibodies, molecular probes and GM-CSF-encoding expression vectors. Clearly, these reagents will be useful in the study of the role of GM-CSF in different pathological conditions, or in vaccination or gene therapy trials.

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SECTION III: GENERAL DISCUSSION

It could theoretically be feasible to completely eradicate amebiasis. The life cycle of *Entamoeba histolytica* is fairly simple compared to that of other parasites, and does not involve any intermediate hosts. Humans and a few other primates are the only reservoirs for the parasite, and this should make the control of the disease relatively easy. Good hygienic practices and the development of adequate water management systems in the endemic countries could, by themselves, reduce considerably the number of cases of amebiasis. However, many developing countries cannot afford sewage treatment and water purification facilities, and their inhabitants are exposed every day to food and water contaminated with *E. histolytica* cysts. Therefore, vaccination against amebiasis appears as an interesting alternative.

Acquired immunity to invasive amebiasis has been achieved repeatedly in animal models of the disease, and it is believed that such immunity also occurs in humans. Recurrence of amebic liver abscess is rare in patients previously cured by chemotherapy (1), and recent epidemiological data obtained with a cohort of children in Bangladesh show a correlation between the presence of amebic-specific coproantibodies and resistance to reinfection (2). Taken together, these observations suggest that a protective vaccine against invasive amebiasis could be possible.

The most promising amebic molecule to be included in an amebiasis vaccine is the Gal-lectin. This surface glycoprotein is one of the major amebic molecules recognized by the serum from amebic liver abscess patients and is therefore very immunogenic (contains numerous T and B cell epitopes). It is also antigenically stable from one clinical

isolate to another (3), and its central role in amebic adherence to intestinal mucus and to target cells makes it a target of choice for the immune system. Furthermore, the native Gal-lectin has been shown to directly stimulate the production of the Th1-promoting proinflammatory cytokines tumor necrosis factor (TNF) and interleukin (IL)-12 by macrophages (4-6), and therefore can possibly act as its own Th1 adjuvant.

It is now generally accepted that a Th1 immune response, or cell-mediated immune response (CMI), is central in host defense and resistance to invasive *E. histolytica* infections. Macrophages activated by interferon (IFN)-γ show amebicidal activity, killing virulent trophozoites *via* the production of nitric oxide (7, 8). DNA vaccination is one of the few immunization strategies that generate a strong Th1 response against a specific antigen (Ag), as well as a high titer of Abs. Thus, we hypothesized that a DNA vaccine encoding the Gal-lectin or a part thereof, could stimulate a strong and protective immune response against invasive disease.

The DNA vaccine we constructed (pCISToGL6, see Manuscript I) encodes a portion of the Gal-lectin heavy subunit (amino acids 894-1081) that includes the carbohydrate-recognition domain (CRD) of the molecule. The nucleotide sequence encoding the Ag was codon-optimized for a high expression in mammalian cells. The vaccine stimulated a Gal-lectin specific immune response in mice, characterized by Ag-specific splenocyte proliferation and serum antibody (Ab) production. The Abs were of the IgG2a isotype, suggesting that a Th1 immune response was induced. In addition to recognizing a recombinant portion of the Gal-lectin 170 kDa subunit, they also bound to the native Gal-lectin on the surface of live trophozoites, and inhibited their adherence to target cells *in vitro*.

The good results obtained with the pCISToGL6 vaccine now set the table for further characterizations and *in vivo* testing. The vaccine can now be tested in the gerbil model of invasive amebiasis to see if it can confer protection against a direct intrahepatic challenge with live trophozoites. Since the coding sequence for the Ag in the plasmid was optimized for gerbil cells, it should lead to a high level of Ag expression *in vivo*. Consequently, the vaccine should stimulate an antiamebic immune response, characterized by Gal-lectin-specific T lymphocytes producing Th1-type cytokines, and by anti-Gal-lectin serum Abs, as it did in Balb/c mice.

The pCISToGL6 plasmid could also be used as a mucosal vaccine. *E histolytica* is a gut-dwelling parasite, and invasion by amebae is first preceded by colonization of the intestinal mucosal surface. An anti-amebic mucosal immune response, consisting of the production, in the intestinal lumen, of secretory IgA (sIgA) specific for amebic surface molecules, is believed to be protective against intestinal amebiasis (2). DNA vaccines can be used to stimulate a mucosal immune response, provided that they are administered at a mucosal surface, and coupled to proper mucosal delivery vehicles, such as liposomes, cochelates and microparticles (9). We believe that pCISToGL6, delivered mucosally (intranasally or orally) in this form, could stimulate the production of anti-Gal-lectin sIgA in the large intestine, which can bind to the CRD of the Gal-lectin and inhibit colonization and/or adherence of trophozoites to colonic mucus and intestinal epithelial cells.

Although our DNA vaccine stimulated a Gal-lectin-specific Th1 immune response, the use of genetic adjuvants should be able to accentuate this response. Cytokines are well-known modulators of immune responses, and DNA encoding Th1 or Th1-promoting cytokines, co-injected with pCISToGL6, should enhance the anti-amebic

cell-mediated immune response. In mice, co-administration of a plasmid encoding IL-12 with a DNA vaccine generally dramatically augments cytotoxic T lymphocyte (CTL) activity and Ag-specific T helper cell proliferation. It also increases the serum IgG2a/IgG1 ratio (enhances a Th1 response), while leaving the overall titer of Ag-specific serum Abs unaffected, compared to injection with the DNA vaccine alone (10-12). In contrast, co-injection with an IL-18 encoding plasmid greatly increases Ag-specific Ab titers without influencing the IgG2a/IgG1 ratio, while increasing CTL activity, and proliferation and IFN-γ production by Ag-specific T cells (10-12). Co-injection with both IL-12 and IL-18 plasmids could lead to an even stronger CMI response, as both cytokines are known to synergize for an even more elevated IFN- γ secretion by T and natural killer cells (13).

While granulocyte/macrophage colony-stimulating factor (GM-CSF) does not drive Th1 differentiation, it can still act as a vaccine adjuvant, mainly due to its differentiation and maturation promoting activities on Ag presenting cells (APC), mainly dendritic cells and macrophages. As GM-CSF acts on a variety of other cell types, including eosinophils, granulocytes and fibroblasts (14), some of these cells could also contribute to the cytokine's adjuvant effect. Co-injection of a GM-CSF plasmid together with an Ag-encoding plasmid generally does not enhance CTL activity, but greatly increases Ag-specific T-cell proliferation in response to the Ag (11, 12). It also dramatically increases Ag-specific serum Ab titers, without significantly influencing the IgG2a/IgG1 ratio.

If the pCISToGL6 DNA vaccine (with or without genetic adjuvants) ever proved effective at conferring protection against amebiasis in humans, it would have several of

the qualities that describe the ideal vaccine. First, being a DNA vaccine, it would be easy, fast and inexpensive to produce, since only a large-scale plasmid preparation from an *Escherichia coli* culture would be required. Similarly, it would be easy to ship from the production place to the vaccination area and easy to store, since plasmid DNA, if kept as a lyophilized pellet, is stable for a long time at ambient temperatures and does not require refrigeration. The vaccine would be easy to administer, either parenterally (intradermally or intramuscularly) with a syringe and needle, or mucosally (orally or intranasally), and would be safe; being a subunit vaccine, it does not contain any immunosuppressive elements (as many whole organism vaccines do), and cannot revert back to a wild-type, pathogenic phenotype, as it has been seen with attenuated live vaccines. Finally, the Gal-lectin-encoding DNA vaccine would stimulate the right kind of immune response (Th1), and would confer protection against a large number of *E. histolytica* strains, as the Gal-lectin is very antigenically stable.

Vaccination against extraintestinal amebiasis is best studied in the gerbil model of the disease. Upon direct intrahepatic injection with trophozoites, these animals develop amebic liver abscesses that resemble those found in human patients. However, very few reagents are available to study immunological parameters in gerbils. Therefore, in the process of developing a vaccine against *E. histolytica* that could ultimately be used in gerbils, and to study the immune response induced by the vaccine in these animals, we had no choice but to produce our own gerbil-specific immunological reagents. As described in manuscripts II, III and IV, we have cloned full length cDNAs encoding gerbil IL-12, IL-18 and GM-CSF. For another study not described in this thesis, we have also cloned the co-stimulatory molecules B7.1 and B7.2 from gerbils, and constructed

expression plasmids encoding them. Mammalian cells transfected with these plasmids expressed the cytokines and accessory molecules in bioactive forms.

The generation of these reagents opens up new possibilities in terms of immunological research in gerbils. The plasmids can be used as genetic adjuvants for DNA or protein vaccination, or in gene therapy trials. The proteins produced by cell lines transfected with them could also be used *in vivo* as vaccine adjuvants, therapeutic agents, or *in vitro* to study their effects on immune cells. Gerbil GM-CSF, with or without IL-4 (the latter has been cloned by Dr. T. Klei's group, University of Louisiana), can now be used on bone marrow precursor cells to generate dendritic cells (DCs) *in vitro* (15, 16). These cells are professional APCs that play a key role in the initiation of primary immune responses, and therefore are extensively studied in the context of vaccination. DC-based vaccination, which consists of pulsing DCs with a protein Ag *in vitro* or transfecting DCs with a plasmid encoding the Ag, and re-injecting the cells *in vivo* (16, 17), is now an avenue than can possibly be studied in gerbils.

The above mentioned cDNA sequences, as well as other partial coding sequences cloned in our laboratory (gerbil TNF, inducible nitric oxide synthase, cyclooxygenase-2, and β-actin), and full-length cDNAs cloned by others (encoding mostly T cell cytokines) can also be used to generate other reagents, such as molecular probes for northern blots or nuclease protection assays, or specific DNA primers and competitors for quantitative or non-quantitative polymerase chain reaction.

Finally, gerbil proteins encoded by the DNA sequences can be produced in a variety of expression systems, purified and used to raise polyclonal or monoclonal Abs against them. For example, Manuscripts II and III report the generation of recombinant

gerbil IL-12 p35 and IL-18, respectively, and of specific antiserum against the proteins. These Abs could then be used to detect the native proteins in immunoblots or by immunofluorescence microscopy or their effect could be studied in *in vivo* or *in vitro* neutralization experiments, to name only a few possible applications.

Gerbils are permissive to a wide range of infections and could therefore be used as animal models for several infectious diseases. The major obstacle to this is, again, the lack of commercially available gerbil-specific reagents. To help the situation, we have recently signed a license agreement with one of the leading pharmaceutical companies specialized in the production of immunological reagents, allowing them to produce and commercialize the gerbil molecules we have identified, as well as Abs raised against them. This will hopefully stimulate an interest in the immunological research community, and promote the gerbil as an animal model.

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APPENDIX

The Thesis Submission Guidelines state that: "If the research for the thesis involved human participants, animal subjects, microorganisms, living cells, other biohazards, and/or radioactive materials, the appropriate compliance certificates must be included as an appendix to the thesis".

The required documents can be found in the following pages.

3

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Kris Chadee TELEPHONE:

   ADDRESS: Institute of Parasitology, FAX NUMBER:

   E-MAIL:

   DEPARTMENT: Parasitology

   PROJECT TITLE: The Gal-lectin in host defence against *Entamoeba histolytica*

2. FUNDING SOURCE: MRC NSERC xx NIH FCAR FRSQ

   INTERNAL OTHER (specify)

   Grant No.: 227-48 Beginning date 01/04/02 End date 31/03/03

3. Indicate if this is

   Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.

   Approval End Date 31/03/02

   New funding source: project previously reviewed and approved under an application to another agency.

   Agency Approval End Date

   New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual". Containment Level (circle 1) 1 2 3 4

Principal Investigator or course director: SIGNATURE date: 25 / 03 / 02 (day / month / year)

Chairperson, Biohazards Committee: SIGNATURE date: 26 03 02 (day / month / year)

Approved period: beginning 01 04 02 (day / month / year) ending 31 03 03 (day / month / year)

D level

ACTION	✓	DATE
P.I.	U	4/11/01
FACC	U	"
RGO	U	"
VET	U	"
DB		

Approved

J. Bingham

McGill University
Animal Use Protocol

☐ **New Application**

☒ **Renewal of Project #** 976-010331

FOR OFFICE USE ONLY

Project #	926
Investigator #	497
Approval End Date	March 31 2002
Facility Committee	ACAR

1. Investigator Information

Principal Investigator: Dr. Kris Chadee Telephone

Department: Parasitology Fax:

Address: Macdonald Campus

E-mail:

Animal Use: Research ☒ Teaching ☐ Specify Course number:

Project Title: The Gal-lectin in host defense against *Entamoeba histolytica*

2. Funding Source

External ☒ Internal ☐

Source(s): Natural Sciences and Engineering Research Council of Canada

Peer Reviewed source: Yes ☒ No ☐ *If no, see instructions - section 2

Awarded ☒ Pending ☐

Funding Period: From: April 2001 To: March 2002

Proposed Start Date of Research: 01/04/01
(Day/Month/Year)

Expected Date of Completion: 31/03/02
(Day/Month/Year)

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name: Dr. Kris Chadee Phone #: Work: Alternative #

Name: Gordon Bingham Phone #: Work: Alternative #

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Animal Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations from the procedures described within.

Principal Investigator/Course Director	Dr. Kris Chadee	Date	February 8/01

Approval:

Chairperson, Facility Animal Care Committee		Date	April 9/2001
University Animal Care Officer		Date	April 11, 2001
Approved period for animal use	Beginning	Ending	March 31, 2002

4/19/APR 10 2001

McGill University Internal Radioisotope Permit

20020225

Permit Holder & Position	Building (Office)		Building (Lab)	Laboratory Classification		Date Issued
KRIS DR. CHADEE, ASSOCIATE PROFESSOR	INSTITUTE OF PARASITOLOGY		INSTITUTE OF PARASITOLOGY	BASIC	**PLEASE POST**	2001/09/01
Department	Room Number(s)	Telephone	Room Number(s)	Telephone		Expiry Date
PARASITOLOGY	B1-14	398-7721	B1-1, B1-2	398-7997		2002/08/31

PERSON(S) APPROVED TO WORK WITH RADIOISOTOPES

Name	Cond(s)	Class(es)	Radioisotope(s)
K. KELLER, TECHNICIAN	2, 4	2, 3	H-3, P-32, C-14
GEOFF HYNES, GRADUATE STUDENT	2, 4	2, 3	H-3, P-32
K. SRINIVAS, GRADUATE STUDENT	2, 4	2, 3	H-3, P-32, S-35
DENIS GAUCHER, GRADUATE STUDENT	4	2, 3	H-3, S-35
DARCY MONCADA, GRADUATE STUDENT	2, 4	2, 3	H-3, P-32, C-14
M. SASAN, GRADUATE STUDENT	2, 4	2, 3	C-14, H-3, S-35
KRIS CHADEE, ASSOCIATE PROFESSOR	2, 4	2	C-14, H-3, P-32
INDRANIL DEY, POST DOCTORAL FELLOW	2, 4	2, 3	H-3, P-32, S-35

GENERAL LICENCE CONDITIONS (OPEN AND/OR SEALED SOURCES)

- The Licence must be posted in the licensee's premises.
- Handling shall be in accordance with the McGill Radiation Safety Manual and with the requirements of the McGill Radiation Safety Commitee.
- The licensee must ensure that all persons mandated to work with radioisotopes be properly trained in radiation safety.
- Laboratories where both radioactive and non-radioactive work are done must have separate areas set aside and clearly identified with radiation warning signs.
- Smoking, eating, drinking, storage of foods or drink and the application of cosmetics and contact lenses are prohibited in areas where radioisotopes are used.
- All procedures involving radioactive materials should be carried out on spill trays or on benches lined with disposable absorbent material.
- Procedures that might produce airborne radioactive contamination should be carried out in a fume hood which is of optimal efficiency. (i.e. free, and to some extent bound I-125)
- When hand or clothing contamination is possible, protective gloves and clothing must be worn.
- After handling radioactive material and especially before leaving the laboratory, personnel must ensure that all parts of their clothes are not contaminated.
- Purchase and disposal of radioisotopes must be documented in a log book.
- For disposal of radioactive waste, consult the McGill Radiation Safety Manual.
- Wipe tests must be made on surfaces and equipment likely to become contaminated with radioactive material. Records of the tests must be properly documented and kept in a log book.
- The Licence must reflect the exact conditions under which radioactive material is used. If changes must be made, contact the Environmental Safety Office at 398-4563.
- The device(s) containing the sealed source(s) listed in this Licence must have an identification label bearing the name and telephone number of the responsible user. A radiation symbol should also be affixed to each device.
- Leak tests must be performed on sealed sources equal to or greater than 50 MBq (1.35mCi).

Approved Unsealed Radioisotope(s) and Location(s)

Isotope	Possession Limit	Stored	Handled
H-3	< 400 MBq (11 mCi)	B1-1	B1-1, A110
C-14	< 10 MBq (270 uCi)	B1-1	B1-1
P-32	< 1 GBq (27 mCi)	B1-1	B1.1
S-35	< 400 MBq (11 mCi)	B1-1	B1-1, A110

Approved Sealed Radioisotope(s) and Location(s)

Permanently Housed Source(s)				*Accessible Source(s)*			
Isotope	Activity	Stored	Handled	Isotope	Activity	Stored	Handled

Personnel Conditions

1. Must be submitted to monthly thyroid bioassays
2. Must wear a whole-body film badge.
3. Must wear an extremity film badge (ex. ring or wrist)
4. Classified as Radiation User
5. Classified as Atomic Radiation Worker
6. Does not work with any isotopes but may be indirectly exposed

Workload Classes

1. Work load < 10MBq (270 uCi) of unsealed radioisotopes in open areas.
2. Work load < 10MBq (270 uCi) of unsealed radioisotopes in a fume hood.
3. Work load > 10MBq (270 uCi) of unsealed radioisotopes in open areas.
4. Work load > 10 MBq (270 uCi) of unsealed radioisotopes in a fume hood.
5. Work with sealed sources.
6. Individual does not work with radioactive sources but normal working conditions involve presence in a room where radioactive material is used or stored.

Joseph Vincelli
RSO & Occupational Hygienist
McGill Environmental Safety Office

Nov 6, 2001
Date

For: Dr. Pierre Belanger, Chairman
McGill Radiation Safety Committee
Dean and Vice Principal (Research)

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SCIENCE

11 October 2002

Our ref: HW/ct/oct 02.169

Denis Gaucher
Institute of Parisitology of McGill University

cc

Dear Mr Gaucher

VACCINE, Vol 20, 2002, pp 3244-3253, Gaucher et al, "Construction and immunogenicity ..."

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11 October 2002

Our ref: HW/ct/oct 02.166

Denis Gaucher
Institute of Parisitology of McGill UNiversity

Dear Mr Gaucher

GENE, Vol 294, 2002, pp 233-238, Gaucher et al, "Molecular cloning and ..."

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Institute of Parisitology of McGill University

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***CYTOKINE,** Vol 14, 2001, pp 177-183, Gaucher et al, "Molecular cloning of gerbil ..."*

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