IN VIVO INDUCTION OF ENDOGENOUS RETROVIRUSES IN BALB/C MOUSE HEPATOCYTES BY SUCCESSIVE TREATMENTS WITH CARBON TETRACHLORIDE AND BROMODEOXYURIDINE

by

HANNAH AYUKAWA

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Department of Biology

McGill University

Montreal, Quebec

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ABSTRACT

The effect of bromodeoxyuridine (BrdUrd) incorporation into cellular DNA on the induction of endogenous retroviruses was examined in vivo. Young BALB/c mice were treated with carbon tetrachloride (CCl_4) to induce liver regeneration, and subsequently received BrdUrd during the period of maximal DNA synthesis. In nucleic acid hybridization studies, the maximum levels of hybridization of both N- and X-tropic retrovirus-specific [^{3}H]-cDNAs were obtained with liver RNA from animals receiving BrdUrd at 40 and 44h post-CCl_4 treatment, and killed on the fourth day following BrdUrd injection. Media from NIH-3T3 (Swiss mouse) and mink cell cultures, infected with liver homogenates from animals treated as above gave significant levels of reverse transcriptase activity. The observations made in the present study show that BrdUrd incorporation into hepatocyte DNA can cause induction of both N- and X-tropic endogenous retroviruses in BALB/c mice in vivo, and such induction is probably a transient event.

RESUME

Les effets, sur l'induction de rétrovirus endogènes, de l'incorporation de bromodésoxyuridine (BrdUrd) dans les hépatocytes du foie en régénération à la suite d'un traitement au tétrachlorure de carbone (CCl_4) ont été étudiés chez des souris BALB/c. D'après les études d'hybridation des acides nucléiques, les niveaux d'hybridation maximums ont été obtenus pour les ADNc tritiés spécifiques des rétrovirus endogènes BALB/c à tropisme N et X avec l'ARN hépatique d'animaux ayant reçu de la BrdUrd 40 et 44 h après le traitement au CCl_4 et sacrifiés le quatrième jour après l'injection de BrdUrd. Dans les milieux de culture de cellules NIH-3T3 (souris Swiss) et de cellules de vison, infectés avec des homogénats de foie d'animaux traités comme ci-dessus, on a observé des niveaux significatifs d'activité transcriptase inverse. Les observations effectuées au cours de la présente étude montrent que l'incorporation de BrdUrd à l'ADN cellulaire peut entraîner l'induction de rétrovirus endogènes à tropisme N et X dans les hépatocytes de souris BALB/c in vivo, et que cette induction est probablement un phénomène transitoire.

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INTRODUCTION

Induction or activation of endogenous retroviruses involves the full expression of the virus genome leading to virus production and the release of infective particles from the host cell. Uninfected cells of BALB/c mice (a low leukemic, inbred strain) contain at least three separate host-range classes of retrovirus genomes integrated into their DNA. B-tropic viruses grow preferentially in BALB/c mouse cells, N-tropic viruses grow preferentially in NIH (Swiss mouse) cells, and X-tropic viruses grow in heterologous cells (i.e. mink, rat, human, etc.). BALB/c cells in culture release all three of these viruses either spontaneously or after treatment with inducing agents. Inhibitors of protein synthesis (e.g. cycloheximide) specifically activate the X-tropic virus (Aaronson and Dunn, 1974); halogenated pyrimidines (bromodeoxyuridine and iododeoxyuridine) induce both N- and X-tropic viruses (Aaronson et al., 1971), and aged and neoplastic cells spontaneously liberate B-tropic virus (Hirsch and Black, 1974).

In vivo, spontaneous release of endogenous viruses is rarely detected in BALB/c mice of less than 6 months of age (Peters et al., 1972). Activation of endogenous N- and X-tropic viruses in young BALB/c mice following skin grafting and immuno-suppression (Hirsch et al., 1975), and of N- and B-tropic viruses

during chemically induced tumour development (Armstrong et al., 1978) has been reported. However, induction of endogenous retroviruses by halogenated pyrimidines in vivo has not been attempted to date. Recombination of endogenous N- and X-tropic viruses has been found to take place in the formation of naturally-occurring leukemogenic virus in AKR mice (Hartley et al., 1977). Such recombinant viruses can be formed only when both parental viruses are produced simultaneously. It is therefore of interest to determine whether BrdUrd incorporation into DNA of cells in vivo causes both N- and X-tropic virus induction.

In the present study the effects of bromodeoxyuridine (BrdUrd) incorporation in hepatocytes of carbon tetrachloride (CCl_4) induced regenerating liver of BALB/c mice on the expression of endogenous retroviruses was examined. The observations presented here show that both N- and X-tropic viruses can be activated in young BALB/c mice by treatment with BrdUrd.

LITERATURE REVIEW

The involvement of viruses in the development of neoplasia was first demonstrated in chickens by the transmission of leukemia by cell-free filtrates of leukemic cells (Ellerman and Bang, 1908), and the induction of solid tumours by extracts isolated from sarcomas (Rous, 1911). The first evidence of such viruses in mice was obtained by Bittner (1936) who found that a factor which is transmitted in the mother's milk causes mammary gland carcinoma in the offspring. Subsequently, the induction of tumours by cell-free filtrates obtained from chemically induced tumours (Zilber, 1946), and transmission of leukemia by extracts of leukemic cells of spontaneous origin (Gross, 1951) have been demonstrated in inbred mouse strains. Leukomogenic viruses are also released during the onset of leukemia in mice exposed to X-rays (Gross and Feldman, 1968), chemical carcinogens (Igel et al., 1969), and specific immunological reactions (Hirsch et al., 1970). These viruses were later shown to belong to a class of enveloped RNA viruses now known as RNA tumour viruses, or retroviruses.

The endogenous nature of the retrovirus genome was suggested by immunological studies which indicated the presence of viral antigens in uninfected embryonic tissues of mice (Huebner et al., 1970). Hybridization of labelled virus-specific DNA copies to

cellular DNA demonstrated the presence of multiple copies of sequences complementary to the viral genome in both normal and transformed cells (Gelb et al., 1973). Further evidence for the presence of endogenous viruses was the spontaneous release of virus from cells following repeated in vitro passage (Aaronson et al., 1969). Subsequently, it was found that treatment of cells with certain chemical agents, halogenated pyrimidines (Lowy et al., 1971), inhibitors of protein synthesis (Aaronson and Dunn, 1974), some mitogens (Greenberger et al., 1975), and 2-deoxy-D-glucose (Prochownik et al., 1976), could greatly increase the frequency of virus activation.

The thymidine analogs iododeoxyuridine (IdUrd) and bromodeoxyuridine (BrdUrd) cause virus production in a wide range of species (Wu, 1977). Virus induction by IdUrd was first demonstrated in AKR embryo cells (Lowy et al., 1971). It has since been shown that established cell lines are more readily induced to release virus than are primary cell cultures, and transformed cell lines are more inducible than nontransformed cells (Rowe et al., 1971; Todaro, 1972).

Studies on the mechanism by which halogenated pyrimidines activate endogenous viruses have shown that the compounds must be incorporated into the DNA to be effective (Teich et al., 1973). The proportion of cells producing viruses decreases if DNA synthesis

is diminished by addition of cytosine arabinoside or serum depletion in the cell culture media during halogenated pyrimidine treatment. Similarly, virus production is inhibited when BrdUrd or IdUrd incorporation is blocked by simultaneous treatment with thymidine. Consistent with these findings is the demonstration that virus induction increases significantly when analog incorporation is enhanced by simultaneous treatment with fluorodeoxyuridine (Teich et al., 1973) and that a period during S phase is the critical time for analog treatment (Besmer et al., 1974).

Levels of virus induction increase when analog treated cells are exposed to visible light, UV, or X-rays (Teich et al., 1973). Since irradiation causes breaks at the site of analog substitution this observation may suggest that BrdUrd incorporation causes DNA strand breaks leading to provirus excision. However, this hypothesis seems unlikely since irradiation alone is rarely sufficient to cause virus induction. At low concentrations, BrdUrd incorporates selectively into intermediate repetitive sequences (Schwartz et al., 1975). Proviral sequences are thought to be integrated in unique (Varmus et al., 1973) or intermediate repetitive (Evans et al., 1974) regions of the cellular genome. It is therefore possible that the critical sequences for BrdUrd incorporation are proviral, a controlling element of

the provirus or a regulatory gene (Besmer et al., 1974). Biochemical studies have indicated that BrdUrd incorporation into DNA condenses chromatin, increasing the affinity of proteins to chromatin (Lapeyre and Bekhor, 1974). Since incorporation into one strand of DNA is sufficient to produce virus (Besmer et al., 1974), it has been suggested that the mechanism by which BrdUrd influences DNA-protein interaction is dependent on the recognition of, or binding to, both DNA strands (Rutter et al., 1973).

The level of viral specific RNA increases in induced cells suggesting that transcription of viral sequences occurs at a faster rate than in uninduced cells (Besmer et al., 1974). IdUrd treatment of AKR-2B cells has been shown to stimulate the overall transcriptional activity, inducing the transcription of unique DNA sequences that are not normally expressed (Chattopadhyay et al., 1979).

A second group of chemicals which induce endogenous viruses are inhibitors of protein synthesis including cycloheximide, pactamycin and puromycin (Aaronson and Dunn, 1974; Cabradilla et al., 1976). These drugs inhibit different steps in protein synthesis and are active only at concentrations that markedly reduce incorporation of amino acids. Neither the inhibitors of RNA or DNA synthesis induce viruses. These findings suggest that it is specifically the disruption of protein synthesis which causes virus

production rather than a general breakdown of metabolic processes (Aaronson and Dunn, 1974). The level of virus specific RNA increases in induced cells, and decreases if the cells are simultaneously exposed to inhibitors of RNA synthesis. This is consistent with a mechanism in which virus expression is regulated by a labile control protein which acts at the transcriptional level, and conflicts with the suggestion that inhibitors of protein synthesis prevent the synthesis of a regulatory molecule which acts post-transcriptionally to stabilize virus specific RNA (Cabradilla et al., 1976).

Other in vitro studies have demonstrated that stimulation of lymphocyte blastogenesis by histocompatibility-associated antigens in mixed lymphocyte reactions or by certain mitogens (eg. lipopolysaccharide) will cause virus induction (Hirsch et al., 1972; Greenberger et al., 1975). However, since lymphoblast proliferation alone is not sufficient for virus activation, it is probably the stimulation of certain specific lymphocytic subpopulations that is required for the induction to take place (Hirsch et al., 1977).

Induction of retroviruses from cultured rat cells has also been reported to take place by treatment with 2-deoxy-D-glucose. Virus release is transient and cell sensitivity to induction was limited to a relatively short period of in vitro growth (Prochownik

et al., 1976).

Certain chemicals are also known to enhance or inhibit virus production in vitro. Glucocorticoids (eg. dexamethasone, hydrocortisone) enhance virus release in cells induced by either halogenated pyrimidines or protein synthesis inhibitors and in cells chronically producing virus (Paran et al., 1973; Dunn et al ., 1975; Ihle et al., 1975). The hormones are active at approximately physiological concentrations and stimulation of virus synthesis requires binding of the steroid to a cytoplasmic receptor protein (Ihle et al., 1975). The hormones alone have no inducing ability. The hormone stimulation of halogenated pyrimidine-induced virus production is not due to enhancement of analog incorporation since maximum stimulation of virus production occurs when the cells are treated 24-48h following IdUrd exposure (Paran et al., 1973). The mechanism of hormone stimulation of virus production is as yet unresolved. Results of one study indicated that its action was post-transcriptional as no increase in virus-specific RNA was detected (Wu et al., 1974). However, in murine mammary carcinoma derived cell lines, dexamethasone enhanced production of mammary tumour viruses by specifically increasing the rate of transcription of viral sequences (Ringold et al., 1977).

A chemical that inhibits RNA tumour virus

production is cordycepin (3'-deoxyadenosine). IdUrd-induced retrovirus release is reduced if cells are exposed to the inhibitor during a critical period, 12-18h after the addition of IdUrd (Wu et al., 1972). Cordycepin treatment reduces the number of cells producing virus approximately 20 fold (Richardson et al., 1976). Cordycepin also depresses the stimulation of virus production by dexamethasone in IdUrd induced cells (Paran et al., 1973). Since cordycepin affects mRNA formation by inhibiting poly-A synthesis it is probable that it blocks virus production directly by preventing the synthesis of the poly-A component of the virus genome (Paran et al., 1973), or indirectly by affecting synthesis of specific proteins required for virus expression (Wu et al., 1972).

Interferon also has the ability to inhibit halogenated pyrimidine-induced virus activation and enhancement of virus production by dexamethasone treatment (Wu et al., 1976).

Some studies have also demonstrated that retroviruses can be activated in vivo. Endogenous virus release in animals often accompanies chemical induction, immunological stimulation, X-ray induction, and spontaneous development of leukemia.

Immunological stimuli that activate endogenous retroviruses include skin graft rejection and graft versus host reactions (for review see Hirsch et al., 1977). During virus activation induced by skin

transplantation, mice are treated with immunosuppressive drugs to prolong graft survival. Xenotropic and/or ecotropic viruses are first detected between 7 and 14 days after grafting in regional lymph nodes and spleens. Thereafter, maximum titres are found in the spleen and are associated with splenomegaly and splenic histological changes (Hirsch et al., 1975). Virus activation takes place in lymphocytes undergoing blastogenesis.

In the graft-versus-host reaction, lymphoid cells from one parental strain are injected into F1 hybrid mice. Antigens from the other parent are recognized as foreign by the donor lymphocytes and a chronic rejection reaction ensues, often leading to development of lymphoid neoplasia (Armstrong et al., 1970). Both xenotropic and ecotropic viruses have been activated in this reaction, and some of these viruses have demonstrable oncogenic potential (Armstrong et al., 1973). The source of activated virus is the injected parental lymphoid cells which undergo blastogenesis when exposed to the other parent's antigen in the F1 mice. Virus replication however also occurs in the proliferating lymphocytes of the F1 mice and most of the tumours that subsequently develop are of F1 origin (Gleichmann et al.,1975).

Treatment of BALB/c mice with pristane (2,6,10,14-tetra-methylpentadecane) has been found to

induce plasmocytomas after a latent period of 6 months (Armstrong et al., 1978). Ecotropic and xenotropic virus titres increase during the latter part of the tumor induction period, in the cell populations in which transformed cells later appear.

It has been found that the murine endogenous viruses induced from the same cells are far from a homogeneous population, and are distinguishable by various criteria including host range, inducibility, immunological reactivity, oncogenicity, and their genomes are independently regulated. For example, in cells of BALB/c mice, there are at least three endogenous viruses of separate host range classes. The ecotropic or mouse-tropic viruses include the B-tropic virus which grows preferentially in BALB/c cells, and N-tropic viruses which grow in cells of the NIH Swiss mouse (Hartley et al., 1970). The third group of viruses which grow in cells of non-mouse origin are termed X-tropic or xenotropic. N- and B-tropic virus genomes share 95-99% homology with one another and a 57-61% homology exists between the X-tropic and N-tropic virus genomes (Callahan et al., 1974). In vivo, expression of N- and X-tropic viral sequences is variable in different tissues; increased levels of expression are seen in dividing tissues such as regenerating liver, uterus, spleen, and embryo as compared to nondividing tissues such as kidney and liver (Mukherjee and Mobry, 1975; Vincent et al.,

1976). Production of viruses also varies with the age of the mice. Virus release before six months of age is rare; N-tropic virus is present mostly before twelve months of age and production of B-tropic virus takes place from aged or neoplastic tissues (Peters et al., 1972). Only the B-tropic virus has been shown to have oncogenic activity (Peters et al.,1973). The differential response of endogenous viruses to inducing agents has indicated that the viruses are independently regulated. Halogenated pyrimidine treatment of BALB/c cells in culture results in the release of N- and X-tropic viruses, but not B-tropic viruses (Besmer et al., 1974). The release of N- and X-tropic viruses is transient; maximum levels of virus production have been reported to be 3-4 days after treatment. Inhibitors of protein synthesis activate only the X-tropic virus, and the induction of virus is also transient, occurring rapidly and decaying more quickly than with halogenated pyrimidine treatment (Cabradilla et al., 1976).

In inbred mouse strains the spontaneous incidence of leukemia varies greatly. Strains such as AKR and C58 have a very high leukemia incidence. In BALB/c and C57 mice, leukemias occur at a moderately low frequency while NIH Swiss mice develop leukemia very rarely (Huebner et al., 1970). Many host genetic factors have been described which affect the incidence and development of neoplasia. These include loci

which determine virus inducibility and infectivity, cell susceptibility to transformation, and the immune response of the animal.

The number and chromosomal location of loci for retroviruses varies from one strain to another. There are two unlinked structural gene loci in AKR mice (Taylor et al., 1971), more than two loci in C58 mice (Stephenson and Aaronson, 1973) and two in BALB/c mice (Aaronson and Stephenson, 1973).

The gene Ind determines the inducibility of N-tropic virus from some mouse strains. NIH mice are Ind- and do not release virus, whereas BALB/c mice are Ind+ and are inducible. Since cells from F1 hybrids (NIH X BALB/c) can be induced to produce viruses Ind is dominant (Stephenson and Aaronson, 1972). The mechanism of Ind action has not been determined.

The Fv-1 locus (located on chromosome 4) controls ecotropic virus infection. There are two alleles, Fv-1N and Fv-1B which determine the permissiveness of cells to N- or B-tropic viruses respectively (Pincus et al., 1971). Resistance to infection is dominant and while the cells from F1 hybrids (Fv-1NB) restrict both types of virus, a class of virus designated NB-tropic is not restricted by either allele (Pincus et al., 1971). Most mouse strains have either one or the other allele, an exception being the feral (wild) mice which lack the Fv-1 gene and therefore are sensitive to all three types of virus (Hartley and

Rowe, 1975). The mechanism of Fv-1 restriction appears to be intracellular since adsorption and penetration of virus into restrictive host cells is not affected (Huang et al., 1973) and cellular extracts of cells with Fv-1N or Fv-1B alleles specifically and reciprocally inhibit replication of B- or N-tropic viruses in permissive cells (Tennant et al., 1974). It has been proposed that the Fv-1 restriction acts at the provirus integration step. Jolicoeur and Baltimore (1976) have demonstrated the presence of nonintegrated provirus in nonpermissive cells and found that both integration of provirus DNA and the level of virus-specific RNA was greatly reduced in resistant cells.

The Fv-2 locus determines the susceptibility of mice to spleen focus formation induced by a NB-tropic component of Friend leukemia virus, the spleen focus forming virus (SFFV) (Lilly, 1970). Mice which are homozygous for Fv-2r are resistant to SFFV induced transformation. Susceptibility is determined by the possession of Fv-2s allele which is dominant.

Another locus that influences MuLV-induced leukemia development is the H-2 (histocompatibility-2) region of chromosome 17. This region influences the threshold dose of MuLV which causes leukemia and the likelihood of recovery from the disease once it occurs (Lilly and Freedman, 1976). Mouse strains possessing the H-2k haplotype exhibit high incidences of

leukemia, induced either spontaneously (AKR, C58), or after inoculation with virus (C3Hf/Bi, C57BR). Strains with other H-2 haplotypes are generally more resistant.

The gene Rgv-1 (resistance to Gross virus) influences the capacity of the host animal to mount an immune response to virus-specified leukemia cell antigens. This gene is associated with the K and/or I segments of the H-2 region. Mouse strains possessing the H-2b haplotype are resistant to the disease and they are efficient at mounting an immune response against the Gross virus (Lilly, 1966).

The susceptibility to Friend virus-induced leukemia is also influenced by the H-2 haplotype. The Rfv-1 gene (resistance to Friend virus) is associated with the D segment of the H-2 region (Chesebro et al., 1974). Mice possessing the genotype H-2b/b are resistant and those with H-2d/d or H-2b/d are susceptible to Friend virus infection. It has been suggested that this gene determines the persistance of viral antigen expression and hence could influence the occurrence of clones of tumour cells no longer expressing certain viral antigens. These cells would therefore escape immunologic attack by the host (Lilly and Freedman, 1976).

Most endogenous viruses induced in vitro or spontaneously released in vivo are nonleukemogenic in the host strain. However, retroviruses are known to

undergo recombination at a relatively high frequency (Vogt, 1971), and it has been shown that many oncogenic viruses are genetic recombinants. The virus now suspected as being the causative agent for leukemia in the AKR strain is a recombinant virus (Hartley et al., 1977). Referred to as the mink cell focus-inducing (MCF) strain, it has the host range of both ecotropic and xenotropic MuLVs and possesses a recombinant ecotropic/xenotropic envelope gene (Elder et al., 1977). Similarly SFFV, a replication-defective transforming virus has a dual host range and has been shown to be a recombinant between a portion of the ecotropic Friend-MuLV and genetic sequences within or closely linked to the env region of a mouse-tropic virus (Troxler et al., 1977). Polytropic recombinant MCF-like viruses have also been detected in leukemic HRS/J (hr/hr) mice (Hiai et al., 1977), in tumours of the reticuloendothelial system of BXH-2 mice (Bedigian et al., 1979) and in thymomas of BALB/Mo mice (Vogt, 1979).

The mechanism of recombination has not been elucidated but it is clear that recombinational events can occur only in cells in which both viral genomes are being replicated. Recent work suggests that the formation of MCF MuLV requires the production of high titres of both ecotropic and X-tropic viruses (Bedigian et al., 1979). In AKR mice, the ecotropic viruses are produced at high titres throughout life.

Xenotropic viruses are not produced until 6 months of age, which correlates with the time of recombinant virus release and leukemia onset (Kawashima et al., 1976). The HRS/J mice, homozygous for the recessive gene hr (hairless mutation), show high incidence of leukemia. These mice produce higher titres of xenotropic virus than the low leukemic hr/+ mice, but the ecotropic virus titres in both strains are comparable (Hiai et al., 1977).

The acquistion of oncogenicity through recombinational events has also been shown to occur during the formation of sarcoma viruses. Sarcoma viruses have the ability to transform cells in vitro and induce solid tumours in vivo. The murine sarcoma viruses have been generated by passage of murine leukemia viruses through mice or rats, and are recombinants of leukemia viral and cellular sequences. In the case of the Kirsten murine sarcoma virus, the cellular sequences have been shown to be from a portion of a rat endogenous virus (Scolnick et al., 1976).

MATERIALS AND METHODS

Mice

BALB/c mice originated from a stock obtained from the Jackson Laboratory (Bar Harbor, Maine). They ranged in age from 2-5 months and weighed 20-30g at the time of treatment. Mice were treated in groups of three and the livers were pooled before RNA extraction or use in the host range determination.

Carbon tetrachloride (CCl4) and bromodeoxyuridine (BrdUrd) treatment

Carbon tetrachloride was administered by an intraperitoneal injection of a 10% solution (w/w) in olive oil. Each mouse received 0.02ml of CCl4 solution/g of body weight. At various times following CCl4 treatment (Table I) the mice received two intraperitoneal injections of 1.5 mg each of BrdUrd, in normal saline (0.9%). Livers were excised on day 3-8 after BrdUrd injection, and stored at -65°C until used. Livers from untreated mice or mice treated only with CCl4 were used as controls.

Determination of BrdUrd levels in blood after intraperitoneal injection

Sixty μCi of [^{3}H]-BrdUrd (15-30 Ci/mmol) was injected intraperitoneally to each animal. At 1, 2, 3 and 4h after injection, blood samples were withdrawn

by opthalmic venous plexus puncture and centrifuged at 400g for 10 min at 4°C. Aliquots of plasma were dried on 0.45mμ Millipore filters and the radioactivity was counted. Blood from untreated mice was processed in the same way as control.

Determination of [^{3}H]-BrdUrd incorporation into DNA of regenerating liver

Following CCl4 treatment each mouse received two injections of 30μCi each of [^{3}H]-BrdUrd at times indicated in Table I. Livers were collected 4h and 4 days after the second [^{3}H]-BrdUrd injection, DNA was extracted, and the radioactivity of DNA samples counted. [^{3}H]-BrdUrd incorporation was estimated as cpm/μg of DNA.

RNA extraction

Total cellular RNA was extracted by the method described by Glisin et al. (1974), with some modifications. Tissues (2-3g) were minced and homogenized in 15 vol of TNE buffer (0.1M NaCl; 0.01M Tris-HCl, pH 7.4; 0.001M EDTA) containing 1% sodium dodecyl sulphate (SDS). The homogenate was extracted with a mixture of 1/2 volume of water saturated phenol and 1/2 volume of chloroform-isoamyl alcohol (24:1) at 65°C, and then at room temperature, followed by several extractions with 1 vol of chloroform-isoamyl alcohol at room temperature. Nucleic acids were precipitated in 2 vol of ethanol, resuspended in TNE

buffer with 1% SDS , and 1g/ml of cesium chloride (CsCl) was added, and mixed thoroughly. Eight ml of this preparation was then layered onto 2ml of 5.7M CsCl in 0.1M EDTA and centrifuged at 134,000g for 12-16h at room temperature. The RNA pellet was rinsed with TNE buffer and resuspended in distilled water at a concentration of 10-15 mg/ml. All RNA preparations had a ratio of absorbance at 260/280nm of approximately 2.0.

DNA extraction

Homogenization of tissue and homogenate extraction procedures were the same as described in RNA extraction method, except that all extractions were performed at room temperature. Ethanol precipitated nucleic acids were dissolved in a buffer containing 0.01M NaCl, 0.01M Tris-HCl (pH 7.2) and 0.001M EDTA. The preparation was adjusted to 0.3M NaOH and incubated for 12-16h at 37°C to hydrolyze the RNA. The samples were then neutralized, dialysed against TNE buffer and then against water, lyophilized, and reconstituted in distilled water.

Synthesis and purification of virus-specific [^{3}H]-cDNA

An endogenous reverse transcriptase reaction described by Benveniste et al. (1977) was used. The reaction mixture contained 0.04M Tris-HCl (pH 7.8), 0.06M KCl, 12mM magnesium acetate, 2mM dithiothreitol,

0.02% (v/v) Triton X-100, 50µg/ml actinomycin-D, 5mM [^{3}H]-TTP (40-60 Ci/mM), 2mM each of dATP, dCTP and dGTP, and purified N- or X-tropic endogenous BALB/c viruses (approximately 0.2-0.4mg virus protein/ml). The reaction was incubated for 90 min at 37°C, deproteinized by phenol-chloroform extraction, dialysed against water and lyophilized. The sample was then applied to a Sephadex G-50 column and fractions containing acid precipitable radioactivity were pooled. The pooled sample was adjusted to 0.5M KOH, incubated at 37°C for 12h, neutralized, dialyzed and lyophilized. The lyophilized material was suspended in water at a concentration of approximately 1000cpm/µl and stored at -20°C until used. These [^{3}H]-labelled DNA products were 98% single stranded, had a specific activity of 1.8×10^7cpm/µg, and represented over 70% of the viral genome at a molar ration of viral RNA to cDNA of 1:1.

RNA-DNA hybridization and analysis

The 300µl reaction mixture contained 0.02M Tris-HCl (pH7.4), 0.06M NaCl, 0.001M EDTA, 0.05% SDS, approximately 1.5×10^4cpm of virus-specific [^{3}H]-cDNA and 5-6mg/ml of total cellular RNA or 0.05mg/ml of viral RNA. Hybridization reactions were heated at 100°C for 10 min and cooled rapidly before incubation at 65°C. Hybridization was measured as the percentage of input [^{3}H]-cDNA counts that remained

trichloroacetic acid (TCA) precipitable after digestion with single strand specific S1 nuclease (Miles Lab). Rot values were calculated as suggested by Britten et al. (1974) and corrected to a monovalent cation concentration of 0.18M. Rot is the product of RNA concentration and length of hybridization reaction and is calculated as nucleic acid absorbance at 260nm x hours of incubation divided by 2.

Determination of host-range of induced viruses

NIH-3T3 and mink cells were grown in Dulbecco's modified medium supplemented with 10% fetal calf serum. Livers from treated and control mice were removed aseptically and homogenized at 4°C. The homogenate was centrifuged at 500g for 10 min at 4°C. The supernatant (2-3ml) was then added to semi-confluent cultures of NIH-3T3 and mink cells which had been pretreated for 1h with medium containing 32µg/ml polybrene. After 2-2 1/2 h of incubation at 37°C the cells were washed and the medium replaced. The cells were grown and subcultured on a regular basis for at least two weeks, before assaying media for reverse transcriptase activity.

Reverse transcriptase assay

Media from cultures to be tested were centrifuged at 10,000g for 15 min and the clarified supernatants were recentrifuged at 150,000g for 30 minutes. Pellets were then suspended in 100µl of 0.02% Triton X-100. Thirty-siven µl of sample was added to 33µl of reaction mixture containing 0.05M Tris-HCl (pH 8.0), 3mM dithiothreitol, 1mM MnAc, 0.03mM EDTA, 0.06M KCl, 0.02 A260 poly(rA), 0.02 A260 oligo(dT) and 0.14µCi of [^{3}H]-TTP. Reactions were incubated for 1h at 37°C, 20µl of saturated sodium pyrophosphate was added, and the TCA precipitable radioactivity was counted.

RESULTS

Availability of BrdUrd in blood after injection

In order to determine the availability of BrdUrd for incorporation into DNA, the plasma level of injected [^{3}H]-BrdUrd was monitored over a 4h period. Figure 1 shows that although the level of [^{3}H]-BrdUrd in the blood dropped sharply between the first and second hour post-injection, a significant proportion remained for at least 4 hours. Since during the present induction studies, treated mice received two injections of 1.5 mg each of BrdUrd at an interval of 4h, BrdUrd was available for incorporation into cell DNA for at least 8 h at an estimated concentration of more than 150µg/ml (based on 2ml blood/mouse). This concentration of BrdUrd was several times more than the amount reported to be required for endogenous retrovirus induction in vitro (Besmer et al., 1974).

Incorporation of BrdUrd into liver DNA

Centrolobular necrosis, comprising 50% of total liver, takes place after CCl4 injection into mice and DNA synthesis begins at 24 h post CCl4 injection. The rate of DNA synthesis is maximal between 40 and 60 h following CCl4 treatment and liver regeneration is complete by the tenth day post-treatment (Schultze et al., 1973). In order to confirm that BrdUrd did get incorporated into cellular DNA, liver DNA was isolated

from mice which had received two [^{3}H]-BrdUrd injections either at 40 and 44 h, 46 and 50 h, or 53 and 57 h after CCl4 treatment and the radioactivity in DNA was counted. Results presented in Table I show that significant incorporation of [^{3}H]-BrdUrd into DNA of CCl4-induced regenerating liver resulted during all three treatment periods. However, the specific activity of labelled DNA decreased 100-1000 fold by the fourth day following CCl4 treatment. This could have resulted from successive cell divisions after labelling, and/or removal of BrdUrd from DNA, as has been reported for regenerating rat liver (Arfellini et al., 1977).

Effect of BrdUrd incorporation on expression of N- and X-tropic viral genomes in BALB/c liver

The hybridization of RNA and labelled viral cDNA probes is an effective technique for the detection of changes in the level of expression of the viral genome. In order to determine the effects of CCl4-induced liver regeneration and BrdUrd incorporation into liver DNA on the transcription of endogenous retrovirus genomes, hybridization of total liver RNA with BALB/c endogenous N- and X-tropic viral [^{3}H]-cDNA was carried out. It was possible that the CCl4-BrdUrd treatment in vivo would have no effect on viral genome transcription, cause a higher expression of the viral genome, or induce the endogenous

retroviruses. If either of the latter two possibilities took place, an increased level of hybridization of the [^{3}H]-cDNA would be expected.

Studies in vitro have shown that induction takes place in only 5-15% of treated cells (Wu et al., 1977). Induction in vivo might involve an even smaller percentage of cells and result in the release of only a small number of viruses. In this case, the number of viral transcripts would be low and greatly diluted by cellular RNAs and therefore saturation hybridization of the viral probe would be difficult to achieve, even with a very high Rot . In such case, an appropriate method of determination of virus induction would be an infectivity assay.

Several sets of hybridization experiments were performed using either N-tropic or X-tropic virus specific [^{3}H]-cDNAs with liver RNA extracted from untreated mice, CCl4 treated mice, and mice receiving both CCl4 and BrdUrd and killed on day 3-8 after BrdUrd treatment. The hybridization values are presented in Table II and Table III and are the maximal levels reached on hybridization curves at the given Rot values.

Table II presents the data obtained from hybridization of liver RNAs to the N-tropic virus specific [^{3}H]-cDNA. In the first set of experiments (performed from August to October 1977), CCl4 treated RNA hybridized to 32%, and BrdUrd and CCl4 treated

RNAs hybridized from 22-49%. The hybridization value for untreated liver achieved at that time was 30% (Mukherjee and Mobry, 1979). These hybridization reactions were carried out to a Rot of 13000-15000, which later experimentation indicated was insufficient for saturation. However, it can be seen that in general those RNAs from mice killed on day 3-5 after BrdUrd gave higher hybridization values than the RNAs from mice killed on day 6-8. Because in vitro studies had indicated that maximal virus release occurs on day 3-4 after BrdUrd it was decided to concentrate efforts on the day 3-5 after BrdUrd time period.

Table II also presents the results achieved in a second set of hybridization reactions performed with the N-tropic probe at a later date (October- December 1978). Hybridization of liver RNAs from CCl4 and BrdUrd treated mice (day 3-5 after BrdUrd), CCl4 treated mice (day 6 and 10 after CCl4) and untreated mice were carried out to higher Rot values (19000-34000). Hybridization of untreated liver RNA reached 40% at Rot 25000 and 42% at Rot 31000. Liver RNA from CCl4 treated animals hybridized 49% (day 6 after treatment), and 41% (day 10 after treatment) at Rot values of 26000 and 25000 respectively. CCl4 and BrdUrd treated liver RNA hybridization levels ranged from 44-53%.

Since hybridization levels reached in the second set of reactions was greater than in the first set, it

can be concluded that the first results were not saturation levels. It is therefore likely that hybridizations with RNAs from CCl4 and BrdUrd treated animals killed on day 6-8 after BrdUrd to higher Rot values would reach the level obtained with untreated liver. However, since this was not tested the possible significance of the lower than normal hybridization levels reached with RNAs from day 6-8 after BrdUrd at the lower Rot values cannot be assessed.

Table III presents hybridization values obtained with the X-tropic virus [^{3}H]-cDNA. In one group of experiments (performed October-December 1977) liver RNAs from CCl4 and BrdUrd treated mice killed day 3-6 after BrdUrd, CCl4 treated mice and untreated mice were hybridized to the virus specific probe. Levels of hybridization for CCl4-BrdUrd treated liver RNA ranged from 33-61% at Rot values of 17000-20000. Untreated liver RNA hybridized 31% (Rot 13000) and CCl4 treated liver RNA hybridized 35% (Rot 20000). When some hybridizations were carried out to higher Rot (October-December 1978) it was found that the value for untreated liver RNA hybridization increased to 38%, for CCl4 treated liver RNA reached 45% (day 6 after treatment) and 41% (day 10 after treatment).

Figure 2 illustrates the most important hybridization curves requiring comparison. It can be seen that total liver RNA from untreated BALB/c mice

hybridized to approximately 42% of the N-tropic and 38% of the X-tropic viral probes. The liver RNA from mice receiving CCl4 treatment alone and collected at day 6 post-treatment, showed increased levels of hybridization with both probes. Hybridization values were 49% for N-tropic and 45% for X-tropic [^{3}H]-cDNA. Maximum levels of hybridization were reached for both viral probes with RNA from animals receiving BrdUrd at 40 and 44 h post CCl4 treatment and killed on the fourth day following BrdUrd injections. This hybridization value was 61% for X-tropic (Fig 2a) and 53% for N-tropic viral probe (Fig. 2b). The hybridization value of 53% was reached at a Rot of 20000 and did not increase at higher Rot (up to 34000); indicating that if viruses were being produced viral RNA was not in sufficient quantity to saturate the probe. Since purified viral RNAs hybridized to 83% with their homologous [^{3}H]-cDNAs, the normalized maximum hybridization values for CCl4 plus BrdUrd-treated liver RNA were 64% for the N-tropic and 73% for the X-tropic probe. These results were consistent with the study of Besmer et al., 1974 in which increased levels of viral specific RNA were detected in induced cells.

However it has been shown that IdUrd can stimulate the overall transcriptional activity of treated cells (Chatopadhyay et al., 1979). Similarly, the observed data could suggest an increased level of

transcription of the viral genome without induction taking place. As it was impossible to distinguish between these two possibilities by furthur hybridization analysis, an infectivity assay was attempted.

Infectivity and host range analysis of induced viruses

Infection of a cell by an endogenous retrovirus results in the integration of a DNA copy of the viral genome into the cellular DNA followed by the replication of virus without any harmful effects on the cell. Viruses bud off from the cell membrane and are released into the culture media. Presence of virus particles in the media can be detected by an assay for the viral enzyme reverse transcriptase (RT).

Since the highest hybridization values were obtained with liver RNA from mice which had received BrdUrd 40 and 44 h after CCl4 and killed on day 4 after BrdUrd treatment, livers from mice receiving this same treatment were tested for the presence of infective retroviruses. Liver homogenates were incubated for approximately 2 h with NIH-3T3 cells (for detection of N-tropic virus), and also with Mink cells (for detection of X-tropic virus). Following this treatment the cell cultures were washed and then grown and subcultured on a regular basis until tests were made for virus production. Exogenous virus particles remain viable and infective for only several

hours at 37°C. Therefore detection of virus particles from the liver homogenate which had not infected cells would not be possible, as cell cultures were not tested until at least two weeks after treatment. This period of time allowed for the spread of viruses to surrounding cells and hence the release of large numbers of viruses into the culture media. Significant levels of reverse transcriptase activity were detected in media from NIH-3T3 cultures infected with three separate treated liver homogenates and from mink cell cultures infected with one treated liver homogenate, indicating that both N- and X-tropic viruses were induced in the treated liver cells. Supernatants of NIH-3T3 and mink cultures infected with liver homogenates from untreated mice and from mice treated only with CCl_4, showed no reverse transcriptase activity (Table IV). Untreated NIH-3T3 and mink cell supernatants also did not show reverse transcriptase activity.

The possibility that unincorporated BrdUrd introduced through the treated liver homogenate to the cell culture caused induction of virus is highly remote for the following reasons. Since the plasma level of BrdUrd decreased rapidly within four hours after treatment (Figure 1); the amount of BrdUrd in the liver 4 days after treatment was most likely significantly below the minimum dose used for in vitro induction (20ug/ml, Besmer et al., 1974). Secondly,

efforts to induce NIH-3T3 cells by halogenated pyrimidine treatment have not been successful to date. Finally, in vitro induction by BrdUrd or IdUrd has been shown to be a highly transient event. The observed high levels of RT activity at 2-4 weeks after treatment indicate that the viral production was not transient, but the result of virus particles infecting and growing in a cell culture.

DISCUSSION

Most in vitro studies on the induction of endogenous retroviruses by halogenated pyrimidines have been carried out with permanent cell lines, which have often acquired abnormal chromosome complements and autonomous growth characteristics. Cells in vitro are exposed to an a different physiological environment than in vivo. They are not under the influence of hormones, immunological surveillance, and extracellular growth control mechanisms. The present study shows that BrdUrd incorporation can cause induction of both N- and X-tropic endogenous retroviruses in hepatocytes of BALB/c mice (Table III), under in vivo physiological conditions.

Several similarities between in vivo and in vitro induction by BrdUrd are evident. N- and X-tropic virus induction from cells in culture occurs over a period of 2-8 days after BrdUrd treatment, reaching a peak at day 4 (Besmer et al., 1974). The present in vivo study showed that N- and X-tropic viruses were released on day 4 after BrdUrd treatment (Table III), and hybridization results suggest that as in vitro, virus production was transient. Hybridization values for both N- and X-tropic viral [^{3}H]-cDNAs with liver RNAs from mice killed at days 6-8 after BrdUrd treatment were close to the levels obtained with liver RNA from untreated animals (Table II). Furthermore,

it has been shown for cells in vitro that incorporation of BrdUrd into DNA synthesized at a restricted time of the synthetic period must take place for the virus induction to occur (Besmer et al., 1974). Since hepatocytes divide somewhat synchronously during regeneration (Schultze et al., 1973), it can be suggested that in vivo BrdUrd incorporation into critical DNA segments takes place 40-48h post-CCl4 treatment.

The observation that liver RNA from animals treated with CCl4 alone hybridized with both viral probes to a greater extent than did RNA from untreated animals (Fig. 2), is consistent with earlier reports of a correlation between increased transcription of endogenous retrovirus genomes and cell proliferation (Mukherjee and Mobry, 1975; Vincent et al., 1976). However, hepatocytes of animals treated with CCl4 alone did not produce viruses (Table III).

Most endogenous viruses released from uninfected cells in vivo or in vitro do not have the ability to cause neoplasia. However, there are examples of endogenous nonleukemogenic viruses acquiring oncogenic activity after being passaged through animals or cells in culture (Haas, 1974; Rapp and Todaro, 1978; Devare et al., 1978). Leukemogenic viruses have also been detected during the onset of leukemia in highly leukemic mouse strains (eg. AKR), in mice exposed to X-rays (Gross and Feldman, 1968) and chemical

carcinogens (Igel et al., 1969), and subjected to certain specific immunological reactions (Hirsch et al., 1970).

Recent studies have indicated that many of the highly oncogenic viruses are generated through recombinational events involving nononcogenic endogenous viruses. The causative agent for leukemia in AKR mice is the MCF (mink cell focus-inducing) virus (Hartley et al., 1977). The MCF virus is polytropic (it has the host range of both eco- and xenotropic MuLVs), and has been shown to possess an ecotropic/xenotropic recombinant env gene (Elder et al., 1977). Similar polytropic recombinant oncogenic viruses have also been detected in other mouse strains that have a high rate of spontaneous neoplasia (Staal et al., 1977; Elder et al., 1977).

Production of both xenotropic and ecotropic viruses occurs in cells of tissues which subsequently become transformed during immunologically stimulated (Hirsch et al., 1975), chemically induced (Armstrong et al., 1978) or spontaneous (Bedigian et al., 1979) neoplasia. There is evidence that it is the release of high levels of xenotropic virus that is crucial in the generation of MCF viruses and leukemia development (Hiai et al.,1977; Bedigian et al., 1979).

In this study it has been shown that both N- and X-tropic viruses can be induced from BALB/c hepatocytes by chemical means. This finding appears

significant since endogenous viruses have been shown to have the capacity to generate oncogenic viruses through recombination, when both viruses are produced in the same cell. It is clear that cells which undergo repeated division to sustain normal functions of various organs might also be vulnerable to BrdUrd-mediated endogenous retrovirus induction and that the in vivo physiological environment does not appear to have a mechanism to prevent induction from occurring. This is particularly important due to the fact that environmental factors, many of them chemical, are now thought to be involved in a significant portion of spontaneous neoplasia in man.

TABLE I

[^{3}H]-BrdUrd Incorporation into DNA of CCl4 Induced Regenerating Liver

[^{3}H]-BrdUrd injection (h after CCl4 treatment)	[^{3}H]-BrdUrd incorporation into DNA: cpm/μg	
	4 h after injection	4 days after injection
40 and 44	2.5×10^4	1.5×10^1
46 and 50	1.9×10^4	1.5×10^2
53 and 57	6.5×10^4	1.5×10^2

TABLE II

Hybridization Levels* of Treated and Control Total Liver RNAs wi
N-tropic Virus-specific [^{3}H]-cDNA

Liver RNA				Hybridization Values			
				Date of Hybridization			
	BrdUrd	Tissue		Aug.-Oct. 1977		Oct.-Dec. 197	
CCl4	Injections	Extraction					
treatment	h after CCl4	days after CCl4 or BrdUrd		% hyb.	Rot (x10^{-3})	%hyb.	Rot (x10
-	-					40	25
						42	31
+	-	6		32	13	49	26
		10				41	25
+	40 and 44	5	3	38	13	46	19
		6	4	49	13	53	20
						51	34
		7	5	24	13	45	19
		8	6	28	15		
		9	7	22	15		
		10	8	21	15		
+	46 and 50	5	3	31	13	49	22
		6	4	31	13	49	22
		7	5	39	13	49	20
		8	6	35	15		
		9	7	22	15		
		10	8	27	15		
+	53 and 57	5	3	32	13	46	22
		6	4	37	13	46	19
		7	5	39	13	45	19
		8	6	32	15		
		9	7	26	15		
		10	8	23	15		

* Based on hybridization values obtained with homologous viral RNAs, the hybridization value attained for each reaction may vary within $\pm$ 2.5 hybridization.

TABLE III

Hybridization Levels* of Treated and Control Total Liver RNAs with X-tropic Virus-specific [^{3}H]-cDNA

Liver RNA				Hybridization Values			
				Date of Hybridization			
				Oct.-Dec. 1977		Oct.-Dec. 1978	
CCl4 treatment	BrdUrd Injections h after CCl4	Tissue Extraction days after CCl4 or BrdUrd		% hyb.	Rot($\times 10^{-3}$)	%hyb.	Rot($\times 10^{-3}$)
-	-			31	15	38	31
+	-	6		35	20	45	26
		10				41	25
+	40 and 44	5	3	33	20		
		6	4	61	19	61	21
		7	5	34	20		
		8	6	38	19		
+	46 and 50	5	3	41	20		
		6	4	38	20		
		7	5	34	20		
		8	6	42	19		
+	53 and 57	5	3	47	17		
		6	4	47	17		
		7	5	43	17		
		8	6	47	19		

* Based on hybridization values obtained with homologous viral RNAs, the hybridization value attained for each reaction may vary within $\pm$ 2.5 hybridization.

TABLE IV

Reverse Transcriptase Activity of Treated and Control Cultures

Cells	Infected with	Expt. #	Time of assay (days after infection)	[^{3}H]-TMP incorporation (cpm)
NIH-3T3	not treated			320*
	untreated liver homogenate	1	15	350
			21	530
		2	15	540
			21	660
	control**liver homogenate	1	14	224
			22	220
	treated***liver homogenate	1	16	205070
			22	240580
			29	332550
			36	453920
		2	16	510780
			29	337380
		3	15	369760
Mink	not treated			135*
	control**liver homogenate	1	14	99
			22	342
		2	14	146
			22	331
	treated***liver homogenate	1	15	2290
			28	510800

* average of six experiments (NIH-3T3), average of three experiments (Mink
** control livers were from animals killed day 6 after CCl4 treatment
*** treated livers were from animals killed day 4 after receiving BrdUrd injections at 40 and 44 h post CCl4 treatment

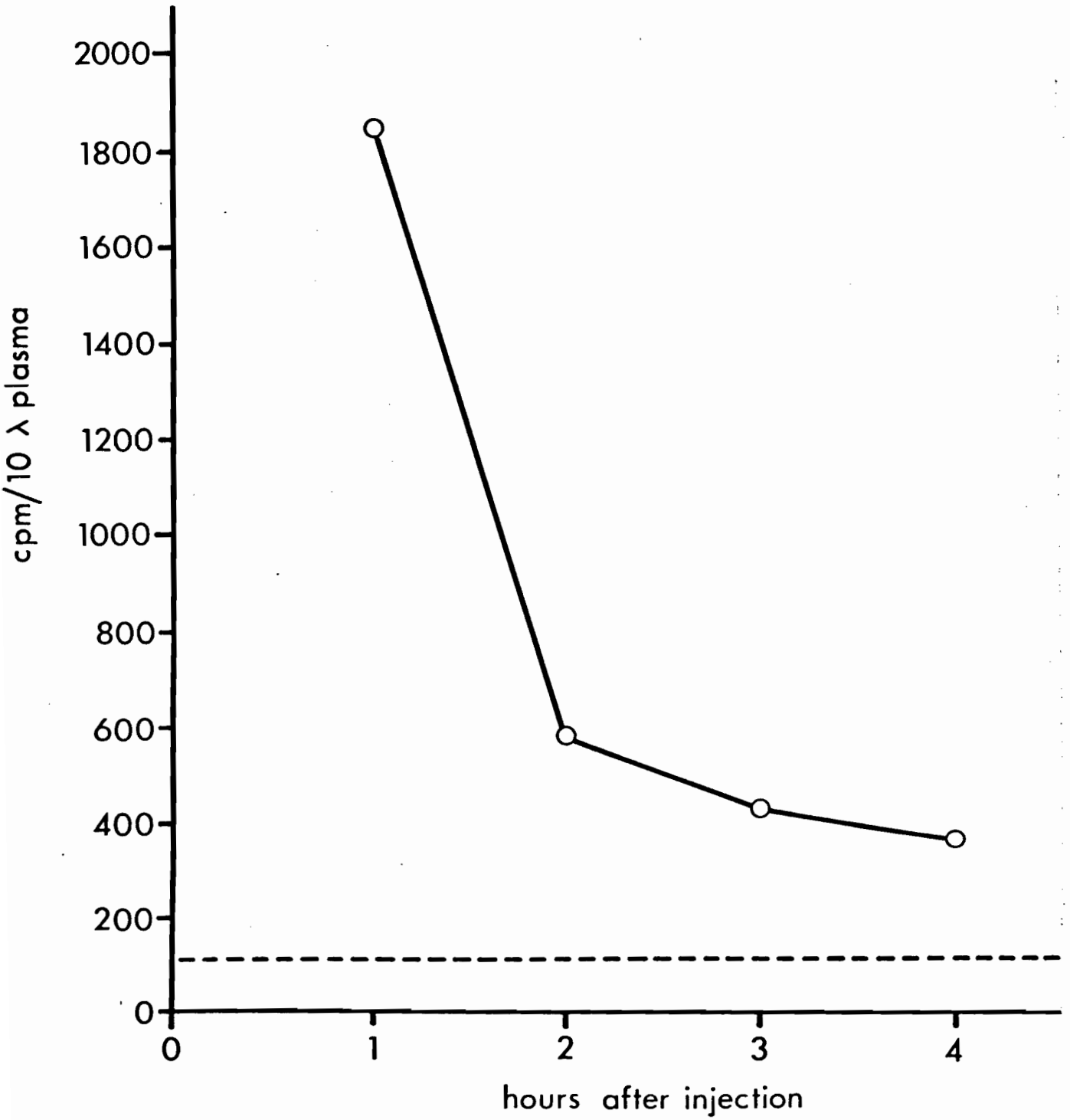
2000
1800
1600
1400
1200
1000
800
600
400
200
0
cpm/10 λ plasma
0
1
2
3
4
hours after injection

FIGURE 1

Plasma levels of [^{3}H]-BrdUrd following intraperitoneal injection.

o----o [^{3}H]-BrdUrd treated mice.

------ untreated mice

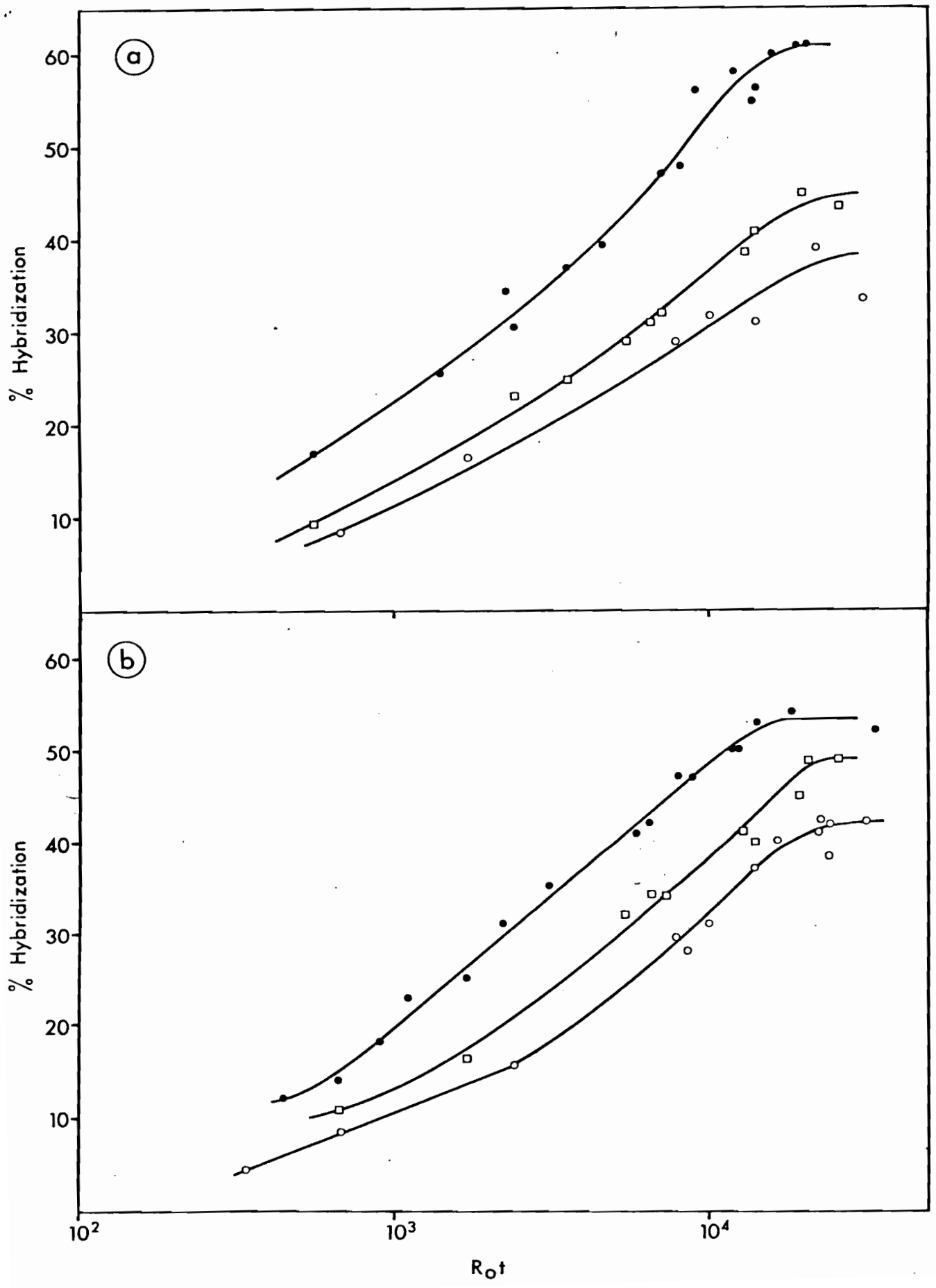
a
b
60
50
40
30
20
10
% Hybridization
10^2
10^3
10^4
$R_o t$

FIGURE 2

Hybridization of a) X-tropic and

b) N-tropic virus-specific [^{3}H]-cDNA

with total liver RNA from:

o----o , untreated mice;

□----□ , CCl4 treated mice (killed day 6 after CCl4);

•----• , CCl4 and BrdUrd treated mice (killed day 4 after BrdUrd injections received 40 and 44 h post CCl4).

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