ABSTRACT

Studies were made of *in vitro* cytotoxicity of human serum on rat bone marrow and spleen cells (xenogeneic system) as well as human lymphocytes (allogeneic system). Cytotoxicity was measured by trypan blue staining and C^{14}-uridine incorporation. Human serum donors were classified into two categories: (a) those without previous exposure to human tissue antigens and (b) those possibly presensitized by means of pregnancy, blood transfusion, hemodialysis or renal allograft. All sera from the first group showed natural xenogeneic cytotoxicity but no allogeneic lymphocytotoxicity. Among sera from the second group, some exerted excessive xenogeneic cytotoxicity (heterophile phenomenon) as well as allogeneic lymphocytotoxicity. Allogeneic lymphocytotoxic antibodies present in renal allograft recipients were found extremely detrimental to grafts when a crossmatch with donor's lymphocytes was positive, but seemed to reveal a high degree of histocompatibility when the test was negative. A panel of allogeneic lymphocytotoxic sera originated from multiparous females was found reliable in determining individual lymphocyte alloantigen profiles.

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To my Mother

ALLOGENEIC AND XENOGENEIC CYTOTOXIC ANTIBODIES IN MAN

by

Jung Hee Oh

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Jung H. Oh

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INTRODUCTION

The experiments to be described in this thesis chiefly concern cytotoxicity of human serum (or plasma) on allogeneic and xenogeneic cells, with relevance to transplantation immunology.

A survey of the literature is made on the whole subject of interactions between sera and cells in mammalian species, as seen in 'HISTORICAL REVIEW'.

The human sera and plasmas used in this work were from persons of both sexes with various medical histories as well as healthy individuals. Among the former were multi-parous women, multi-transfused patients, non-dialysed uremic patients, uremic patients with repeated hemodialysis and renal allograft recipients. It seems appropriate to stress here that all human subjects studied can be classed into 2 groups: those without history of exposure to human tissue antigens and those who have had this sort of exposure and might therefore have been sensitized to human antigens.

Cells used were human peripheral lymphocytes and rat bone marrow and spleen cells.

Cytotoxicity was measured by two parameters: trypan blue staining and C^{14}-uridine incorporation rate. The former was done according to the conventional technique. The latter was, however, considerably modified and simplified by the candidate from the

techniques reported by other investigators.

Xenogeneic cytotoxicity was investigated before allogeneic cytotoxicity. It was the former which provided the candidate with much of his interest in cytotoxicity phenomenon and led him into further research into the latter system.

The experiments concerning xenogeneic cytotoxicity of human serum and plasma on rat bone marrow and spleen cells were directed to study (a) relative cytotoxicities of sera (or plasmas) from various categories of human subjects and (b) factors modifying cytotoxic reaction, including relative potency between serum and heparinized plasma from the same individuals. A number of horse anti-human lymphocyte sera were also tested for their in vitro cytotoxicity on human lymphocytes.

The experiments concerning allogeneic cytotoxicity exclusively dealt with lymphocytotoxicity and constitute the latter half of this work. Studies were made of (a) incidence of allogeneic lymphocytotoxic antibodies in varying categories of individuals, (b) the value of crossmatch between recipient's serum and donor's lymphocytes prior to renal transplantation and (c) specification of antisera with respect to seven defined lymphocyte antigen groups.

The candidate considers the following elements in this thesis to be contributions to original knowledge.

a. Methodological

1) Use of the rate of incorporation of radioactive nucleoside (C^{14}-uridine) into nucleic acid (C^{14}-RNA) as a measure of cell viability.

2) Instantaneous disruption of cell membrane using sodium lauryl sulfate buffer in order to facilitate the separation of macromolecules from their precursors upon subsequent treatment with trichloroacetic acid.

3) Introduction of "mixed cell incubation" as a rapid method to screen human sera for the presence of allogeneic lymphocytotoxic antibodies.

b. Experimental

1) Demonstration of heterophile cytotoxicity in sera of some human subjects who have been exposed to human tissue antigens.

2) Pretransplant crossmatch of recipient's serum with donor's lymphocytes in order to avoid immediate type of renal allograft rejection due to preformed cytotoxic antibodies.

3) Specification of allogeneic antisera with respect to defined lymphocyte antigen groups, using a panel of pretyped lymphocytes.

HISTORICAL REVIEW: ACTION OF MAMMALIAN SERUM ON ALLOGENEIC AND XENOGENEIC CELLS, RELEVANT TO TRANSPLANTATION IMMUNOLOGY

A historical review of the action of mammalian serum on mammalian cells will be made under the following classification based on genetic relationship between serum and cell donors.

The following list is intended to cover those interactions which have more or less a bearing on transplantation immunity in mammalian species, particularly in man. Therefore, a few other possible combinations of serum and cell such as those related to anti-microbial immunity or autoimmunity are not primarily considered except when they have some significance in transplantation immunology.

A. Normal serum: Natural antibodies
   1. Action of normal serum on cells of same species.
   2. Action of normal serum on cells of other species.

B. Immune serum: Acquired antibodies
   1. Action of immune serum on cells within same species.
   2. Action of immune serum induced in other species on cells of immunizing species.
   3. Action of immune serum induced in same or other species on cells of species different from immunizing species.

The above list is represented schematically in the following diagram.

SCHEMATIC REPRESENTATION OF SERUM-CELL INTERACTIONS IN MAMMALIAN SPECIES

Serum \ Cell		Same Species: Individual (I)	Same Species: Other Individual (II)	Other Species (III)	Microbes (IV)	Example
A. Normal Sera (natural Antibodies)	1	I ⟵ II			IV	Human Red Blood Cell Isoagglutinins
	2	I ⟵ III			IV	Heterotoxins
B. Immune Sera (Acquired Antibodies)	1	I ⟵ II				Allogeneic Lymphocytotoxins (Tissue Typing Sera)
	2	I ⟵ III; II				Xenogeneic Antilymphocyte serum
	3	I ⟵ II; IV				Heterophile Antibodies
		I ⟵ II; III				

Legend: ..... Possible Sensitization (not determined)

---------- Active Sensitization

_____ Immune Response

Before elaborating on each category of these reactions, a few points should be mentioned. First, the terms used in this thesis concerning genetic differences were originally proposed by Gorer et al [87] and adopted by Russell et al[224]. Thus, the following relations should be noted:

Old terminology	New terminology	Meaning
Autologous	Autologous	Same individual
Isologous	Isogeneic	Identical genetic make-up
Homologous	Allogeneic	Different genetic make-up within same species
Heterologous	Xenogeneic	Between different species

Second, although the primary interest of this candidate resides in immune cytolytic phenomenon, this review is not restricted to cytolysis but covers other manifestations of interaction of serum and cells in vitro, e.g. agglutination.

In the following, each topic in the above list is reviewed individually.

A. Normal serum: "Natural Antibodies".

The sera of most animals contain what appear to be "natural antibodies"; that is, they are not apparently formed by any known

antigenic stimulus. These antibodies can react with cells from same or other species and sometimes with both.

1. Action of normal serum on cells of same species: "natural allo-antibodies (iso-antibodies)":

In normal human sera, natural antibodies are found which react with the ABO (144, 145), Lewis (189) and P (146) antigen systems of the erythrocyte (122, 289), and with the ABO and perhaps some antigens of platelets (92). These antibodies are not lytic or complement fixing (94, 210). No natural allo-antibody to the erythrocyte has been reported in any mammals other than the human.

As far as leukocytes are concerned, however, no natural allo-antibody has been demonstrated in any mammalian species. In view of the failure to demonstrate natural allogeneic leukocytotoxic antibodies in mammalian sera, it seems interesting to note that a skin allograft to unsensitized subjects undergoes the "first set rejection" (as opposed to the "second set rejection"), usually living for one to two weeks (215) in human and for about one week in rabbit (168). If certain straightforward requisites are fulfilled, such as provision for an adequate blood supply, the complex process underlying the active rejection of an allograft by its host usually requires at least a few days to become manifest, thus

ruling out any sort of "built-in" or "ready made" response existent in the previously untreated recipient (223).

2. Action of normal serum on cells of other species:

An in vivo experiment to show toxicity of normal xenogeneic serum injected directly into animals was carried out as early as in 1875 by Landois (143). Since then, there appeared numerous reports to describe a lethal effect of normal xenogeneic serum in various combinations of mammalian species (249).

Various hypotheses for the cell injury produced in vivo by normal xenogeneic serum were given, such as anaphylaxis (303) and hemolysis (70). Only Delaunay et al (52) seem to have related in vivo toxicity with in vitro toxicity, as reviewed below. Lambert and Hanes (142) in their pioneer tissue culture studies of 1911, noted that fresh normal sera from goats, dogs and pigeons were toxic to rat spleen, rat sarcoma and mouse carcinoma cells. In 1917, Pappenheimer (200) demonstrated varying degrees of toxicity by normal sera of human, horse, rabbit and guinea pig on rat thymus cells. Since then, many investigators (22, 23, 29, 65, 114, 162, 179, 288, 291) also made observations on the in vitro xenogeneic cytotoxic phenomenon. Among them were Lumsden and Kohn-Speyer (162) who showed that mouse cells were killed in tissue culture by fresh serum from rabbit, chicken, guinea pig,

sheep, goat and human. And they proposed the term "heterotoxins" for the toxic factors in normal serum.

The existence of such toxins was not, however, recognized by all and, indeed, some workers (97, 98, 99, 167, 188) reported certain evidence to the contrary. In addition, the common usage of xenogeneic sera in tissue culture media indicated the extent to which these xenogeneic effects were ignored.

In 1961, Terasaki et al (249) extensively studied comparative effects of individual xenogeneic sera on lymph node cells of common laboratory animals and stated that certain combinations were considerably more toxic than others. In general, human, chicken and rabbit sera were harmful to cells of other species tested. Rat sera were markedly toxic only to chicken lymphocytes and, on the whole, mouse and guinea pig sera did not greatly affect lymphocytes of other species. However, because of individual differences and probable strain differences, generalizations could not be made about the incompatibility of given combinations of serum and cells.

Several important aspects concerning the nature of natural xenogeneic cytotoxicity were clarified by the work of Landry et al (149). They showed that normal human serum depleted individually of four components of complement (C'1, C'2, C'3, or C'4) lost its ability to kill mouse Sarcoma 37 cells. Also, fixation of complement by a preformed antigen-antibody complex destroyed cytotoxic

activity as did binding of divalent Ca and Mg ions. The activity, however, was restored when sera depleted of C'3 or C'4 were mixed. From these experiments, it was concluded that complement was one of the factors responsible for xenogeneic toxicity. The other factor was shown to be absorbed from human sera by Sarcoma 37 as well as by other tumors and normal tissues of mouse. It could be eluted from the Sarcoma 37 cells. This factor was heat labile, being destroyed by heating at between 56 and 60°C for 30 minutes. Absorption of properdin did not affect the cytotoxic activity of normal serum. From these experiments, Landry et al (149) concluded that this second factor in xenogeneic toxicity was an antibody and that the whole system was an antibody-complement system.

The role of natural xenogeneic antibodies may be reflected on the behavior of xenografts. In general, a xenograft among mammalian species is met with a more rapid and violent reaction than an allograft. Skin grafts among rodent species, for example, are scarcely able to become united to their beds and vascularized before the rejection begins to take effect. Cellular infiltration is less obvious, and the vascular component of the reaction is more marked (224). In fact, Loeb (161) and Algire et al (4) suggested that free antibody may be capable of destroying cells transferred to a xenogeneic environment without the participation of cells. It

would be interesting to examine whether there is a correlation between the degree of in vitro serologic reaction and the rapidity of xenograft rejection among mammalian species.

Although these natural antibodies, allogeneic as well as xenogeneic, are found in mammalian sera in early life and are not apparently due to any known antigenic stimulus, there have been a few possibilities reported to suggest that natural antibodies are in fact acquired. The possibilities are: (i) Natural antibodies could be passively acquired through colostrum from the mother, which has been actively sensitized to related antigens [112]. (ii) They may be immune antibodies formed in response to infectious agents which contain antigens similar to those in mammalian cells [66, 122, 196]. The latter possibility was particularly well illustrated by Fedoroff et al [66] in their experiments with human serum acting on mouse cells and certain bacteria. They found that normal human serum was lethal to Strain L mouse cells and also reacted with Salmonella typhosa and Escherichia coli. Furthermore, incubation of normal human serum with those bacteria rendered the serum almost non-toxic to the mouse cells. These findings led the investigators to postulate that anti-mouse antibodies could have been produced by previous contact with the bacteria, that is, they were immune antibodies resulting from cross-reacting antigenic stimulation.

Moreover, experiments in which chicks, rats, guinea pigs or piglets are reared in "germ free" environments, have shown that such animals do not develop "natural antibodies" until they come into contact with bacteria or their products (112).

Judging from these observations, it is quite possible that natural xenogeneic antibodies found among mammalian species are in fact heterophile antibodies which react with antigenic determinants common to mammals and microorganisms. This possibility, however, has to be based on an assumption that an antigenic stimulus once begun this way could be continued throughout life. The whole subject of heterophile antibodies is dealt with later in this review.

On the other hand, however, there have been reports describing differences in physico-chemical properties between antibody present in normal serum and that contained in immune serum. Landy (149, 150) stated that concentration of antibodies in normal serum is very low compared to those in immune serum and that natural antibodies are heat-labile, being inactivated between 56 and 60°C. The latter criterion, however, cannot be taken too seriously in view of the findings that some natural antibodies are stable at this temperature (162, 249) whereas some immune antibodies are destroyed at the same temperature (101, 102). It was also mentioned that natural antibodies, particularly in young animals or infants, were mostly found to occur in 19 S gamma

globulins [53, 112], whereas immune antibodies are usually 7 S gamma globulins [173].

After all, it has to be said that much obscurity still surrounds the genesis of natural antibodies.

B. Immune Serum: "Acquired Antibodies"

This section deals with the action of sera from actively immunized individuals on cells of same or other species. Thus, any given serum referred to in this section is an "immune serum" or "antiserum" as distinct from a "normal serum" which has been dealt with in the preceding section.

Three different combinations of antiserum and cell are considered, all of which have some relevance to transplantation immunology.

1. Action of immune serum on cells within same species:

This subsection concerns the effect of antiserum induced within a species on cells from individuals of the same species including the immunizing donor. Accordingly, this is intimately related to the humoral aspect of allograft immunity.

The general conclusion that humoral antibodies might not play a major role in allograft rejection [171, 174, 237] has been based on three areas of experimentation: (i) the early failure of attempts to demonstrate in vitro immune reactions in sera of allo-

graft recipients (5, 97, 170, 180), (ii) the experiments by Algire, in which allografts enclosed in millipore chambers, permitting passage of humoral but not cellular elements, survived for long periods, even when placed in a previously immunized host (2, 4) and (iii) the repeated failure to transfer allograft sensitivity by large doses of serum(16, 17).

However, many later publications reported results contrary to the above.

Firstly, antibodies could be demonstrated, in vitro, following immunization with allogeneic tissues in mice (6, 241), rats (199), rabbits (96, 131, 247), dogs (244), guinea pigs (280), chickens (248), men (281), and many other species including reptiles (106) and fish (221). Antibodies were also demonstrated, in vitro, after immunization with certain tumor cells (80, 89, 103, 163, 183, 231, 292). Thus it is now generally recognized that antibody production is probably an almost invariable consequence of allotransplantation. Furthermore, antibodies have been demonstrated in a certain portion of humans following blood transfusion (45, 204, 266) or pregnancy (205, 252, 273). The antibodies thus formed are, of course, directed against isoantigens present in donor tissue but absent from the host, and may be detected by hemagglutination (88), leukoagglutination (6), hemolysis (105), or a variety of cytotoxic reactions (225). Particularly, in the demonstration of cytotoxic antibodies the importance of complement can not be overemphasized (253).

Secondly, subsequent studies of certain types of allografts in diffusion chambers have rendered the earlier findings of Algire [2, 4] less conclusive. Apparently, humoral antibody and complement penetrate such chambers with difficulty, and when they do so in appropriate levels some allografts are destroyed [10, 77, 278]. Presumably, not all grafted cells are equally susceptible to destruction in a millipore chamber [3].

Thirdly, regarding the passive transfer of allograft sensitivity, a number of different grafted tissues have been studied. Particularly noteworthy are experiments done by Voisin and Maurer [277] and by Steinmuller [239]. Voisin and Maurer [277] showed that sera obtained from guinea pig A sensitized to guinea pig B caused, upon injection into guinea pig C, some degree of accelerated rejection of skin grafted to guinea pig C from guinea pig B. Steinmuller [239] reported that antiserum from BN rats, harvested at a time of maximum rejection of Lewis skin grafts, produced accelerated rejection of Lewis grafts in normal BN rats.

These findings all seem to bring arguments in favor of the role of humoral antibodies in the rejection of allografts.

The significance of allogeneic antisera is not limited to the allograft rejection. In recent years these antisera have been used for serologic histocompatibility testing in humans as they recognize, in vitro, transplantation antigens present in leukocytes [51].

Dausset [48] and van Rood [268] recently reported a review on the work concerning the relationship between leukocyte antigens and transplantation antigens. It was Medawar [169] who opened this field in 1946 with his classical study showing that the intradermal injection of buffy coat evoked allograft sensitivity in rabbits. In 1961, Rapaport et al [219] and Friedman et al [76] confirmed these findings for humans. The reverse situation was also proven by Amos [6] who demonstrated the presence of leukoagglutinins after skin grafting in mice, and by Gorer et al [83] who established the occurrence of cytotoxic antibodies against H-2 antigens after rejection of grafts. Subsequently these findings were shown to hold for humans as well by Colombani et al [43] using antiglobulin consumption test, by van Rood et al [272] using leukoagglutination test and by Walford et al [283] and Terasaki et al [252] using lymphocytotoxicity test.

If leukocytes carry transplantation antigens, the next step is to classify leukocyte antigen groups and to examine the relation of the defined leukocyte groups to the transplantation antigens. Dausset [47] was first able, by immunizing a number of recipients with leukocytes from a single donor, to produce leukoagglutinin which seemed to recognize a single antigen called "Mac". Later when it was realized that most of the leukocyte antibodies are frequently formed after pregnancy [46, 205, 269, 273], van Rood et

al [267, 271], through chi-square analysis, devised a method to select the sera most likely to recognize single antigens, using leukoagglutination test. Soon Terasaki et al [255], using lymphocytotoxicity test, examined 154 sera on a panel of lymphocytes from 48 to 194 unrelated individuals. This enabled them, through computer factor analysis, to classify most of the sera into 7 major groups and, through regression analysis, to assign provisional types to individual lymphocytes of the panel. With regard to the relationship of defined leukocyte groups to stronger kidney transplant-antigens, Terasaki et al [258] analyzed the survival of renal allograft recipients matched for major leukocytes groups in comparison to mismatched recipients and concluded that although the survival in his series was associated with matching in marginal limits, many hazards which influence survival especially in the first 3 month period might be making it difficult to demonstrate the association. He then suggested that further experience would be necessary to determine the association more conclusively.

2. Action of immune serum induced in other species on cells of the immunizing species:

Pappenheimer [200] reported, in 1917, that sera of rabbits immunized against rat thymus cells or human tonsil lymphocytes were strongly toxic to cells used for immunization. Following this, many experiments concerning sensitization of xenogeneic animals

were done using mainly neoplastic cells (15, 41, 42, 59, 71, 109, 158, 167, 295).

In transplantation research, xenogeneic immunization has been attempted for two quite opposite purposes: one, to produce xenogeneic antisera which cause accelerated rejection of allografts when given to the immunizing species, and the other, to produce xenogeneic antisera which prolong allograft survival when given to the immunizing species.

Examples of the accelerated rejection of allografts are found in the experiments of Voisin and Maurer (277). They sensitized a rabbit to the skin of guinea pig "A" by subcutaneous inclusion. The skin of guinea pig "A" was then grafted to guinea pig "B" which had previously received the immune rabbit serum. The graft was rejected in an accelerated fashion. A similar type of rejection was observed when guinea pig "B" received a skin graft from guinea pig "C". These results were thus indicative of the occurrence of anti-guinea pig skin antibodies, species specific as well as individual specific, in the sensitized rabbit.

On the other hand, xenogeneically induced antisera intended to protect allografts are primarily directed against lymphoid cells, and are therefore called anti-thymus or anti-lymphocyte serum. Metchnikoff (175), in 1899, immunized guinea pigs by subcutaneous injection of rat spleen or lymph node emulsion, yielding antiserum

in the guinea pig which agglutinated and lysed the rat leukocytes. Later, Pappenheimer [201], in 1917, showed that rabbits which had received intravenous injection of rat thymus cell suspension yielded sera with both leukoagglutinative and leukocytotoxic properties and that absorption by rat red cells did not reduce the antileukocyte activity of the rabbit antiserum.

However, the use of xenogeneic antilymphoid serum for prolongation of allograft survival is based on relatively recent studies by Waksman et al [279], Woodruff et al [297, 298, 299], Gray et al [90], Jeejeebhoy [119] and Monaco [185] on skin allografts in small animals such as guinea pigs, rats and mice. Antilymphocyte serum was also effective in prolonging canine renal allografts as shown by Hinchey et al [107], Abaza et al [1], Huntley et al [113], Monaco et al [184] and Shanfield et al [228]. Recently, the use of antihuman lymphocyte serum has been described. Starzl et al [238] have claimed that antihuman lymphocyte serum produced in horses prolongs kidney allograft survival in man. Monaco et al [186] reported that antihuman lymphocyte serum produced in rabbits also causes modest prolongation of skin allograft survival.

There has been no conclusive proof as to the mode of action of antilymphocyte serum in vivo in spite of intensive study. Gray et al [91] described a state of lymphoid depletion in mice given antilymphocyte serum and stated that the immunosuppression seemed to

last only as long as the lymphoid depletion persisted. Monaco et al [186] reported a similar finding in man, that is, human subjects given a rabbit anti-human lymphocyte serum showed transient lymphopenia associated with moderately prolonged abolition of delayed type of skin reactions and modest prolongation of skin allograft survival. But, Levey and Medawar [154] first proposed the so-called "blindfolding" hypothesis, according to which antilymphocyte serum acts by surrounding lymphocytes with a protein coat that interferes with antigenic recognition. However, their ensuing experiments [155, 156] indicated that antilymphocyte serum impairs immun ological capability of lymphoid populat ions through at least one cellular generation, and that it acts primarily on peripheral lymphocytes, affecting lymph nodes in proportion to their population recruitment from circulating lymphocytes. These findings cast grave doubt on the blindfolding hypothesis, while leaving open the possibility that antilymphocyte serum acts by a mechanism of "sterile activation" [156].

3. Action of immune serum induced in same or other species on cells of species different from immunizing species: "Heterophile phenomenon".

The occurrence of cross-reacting antigens in the cells of unrelated species was originally described, in 1910, by Ehrlich et al [61] who demonstrated that sensitization of rabbits with ox erythro-

cytes induced the developments in the rabbits of sheep-and-goat-hemolysins as well as ox-hemolysins. And, in 1911, Forssman [74] observed that when watery suspensions of certain organs of guinea pig were injected into rabbits, a marked augmentation of the normal sheep cell hemolysis took place. These observations opened a line of intensive investigation on the occurrence and distribution of cross-reacting antigens between many different species of mammals. Soon, Forssman antigens were detected in the tissues of, besides guinea pig and sheep, many other mammals including mouse, hamster, goat, cat, dog, pig, horse and cow. However, the antigens were absent in other mammals including rat, monkey and chimpanzee. Man was considered a Forssman-negative species, although Forssman-like antigens were described in subjects of blood groups A and AB [34].

Heterophile antigens other than the Forssman type were also reported in mammals, pre-eminently in erythrocytes and occasionally in other tissues as well. The antigens of this type were found in ox, goat, and sheep [61, 75, 187]; in horse and donkey [148]; in rhesus monkey and swine, and in horse and rat [147]; in swine, ox and human erythrocytes of group A [296]; in cat and horse [140]; in dog, swine, cat and man [137]; and finally, in man and rhesus monkey [33]. Buchbinder [34] stated that some of these antigens might be found to overlap.

In 1961, Brent et al [32] extended the heterophile antigen concept to transplantation biology by their description of serologic

cross-reaction between pneumococcal (Type XIV) polysaccharide and the H-2 transplantation antigen of mouse. In addition, Rapaport et al [39, 211, 212, 213, 214] demonstrated that heat-killed group A streptococci induced, in mice, rats, guinea pigs, and rabbit, a state of hypersensitivity to skin allografts, which was indistinguishable from that resulting from pretreatment of the recipients with allogeneic tissues, providing evidence that stimulation by xenogeneic antigens may be operative in conditioning host responses to allotransplants. Rapaport et al [211, 216, 217, 218] extended this concept of heterophile antigens to an assessment of serological components possibly involved in allograft responses in man. They observed that rejection of skin allografts was associated in human recipients with the development of agglutinins against sheep, guinea pig and rat erythrocytes [211, 216, 217], and reported similar observations in individuals subjected to various other modes of sensitization, namely, leukocyte injection, pregnancy or renal allograft [218]. Particularly in the latter experiments, the occurrence of peak antibody titers very often coincided with allograft rejection, leading the authors to propose the use of heterophile hemagglutination test as a possible warning system for the diagnosis and prompt management of rejection crisis in clinical organ transplantation, where antigenic differences between donor and recipient are capable of evoking this type of response.

Having reviewed the historical mile-stones leading to the present knowledge concerning the interaction between serum and cells in mammalian species, a brief description seems to be in order with regard to the modes of the reaction.

Obviously, the in vivo cell-serum reactions are not subject to a direct observation. On the other hand, the in vitro reactions can be classed into 3 main types: simple cell-antibody combination, agglutination and cytolysis (112). The first type of reaction may occur in case of incomplete antibodies and can only be detected by somewhat complicated techniques such as antiglobulin consumption test or fluorescent antibody technique.

Since data for this thesis exclusively concern the cytolytic reaction, a brief resumé on the mechanism of immune cytolysis seems pertinent.

Immune cytolysis is in essence a cell membrane damage by antibody and complement (193). The term complement refers to a set of serum factors which, together with antibodies, have the capacity of causing lysis of cells. In addition to causing direct cell lysis, it is capable of engaging in a number of non-cytolytic reactions which may lead to indirect cell injury.

Through the work of several groups of investigators, it has become apparent that the complement system comprises at least 11 different serum factors: C'1q, C'1r, C'1s, C'2, C'3, C'4, C'5, C'6, C'7, C'8 and C'9 (301). These factors can be separated from

each other and some of them have been isolated in highly purified form. Muller-Eberhard et al [193] described the present concept of the mode of action of complement as below.

When a cell is attacked by specific antibody and complement, it rapidly undergoes drastic morphological changes [79] which result in cell death. When examined with the electron microscope, it becomes apparent that the outer cell membrane has sustained characteristic ultrastructural lesions. These lesions, which were first described by Borsos et al [24], have been interpreted to constitute membrane holes.

The above immune cytolytic reaction begins with the attachment of C'1 (q, r and s) to antibody on the cell surface [152, 194]. Borsos and Rapp [25] have shown that a single bound antibody molecule of γM type is sufficient for the binding of the first component. The bound C'1 first mediates binding to the cell surface of C'4 [192] then binding of C'2 [13, 233, 243]. Next, C'4 and C'2 together mediate binding to the cell surface of C'3 [191]. Thereafter, C'5, C'6 and C'7 [300], after being activated by the preceding components, act on a membrane site, modifying it so that it becomes susceptible to the action of the last two complement components, C'8 and C'9 [159], which ends with the death of the cell.

Since data presented in the candidate's thesis concern solely humoral aspect of transplantation immunity as distinct from cellular immunity, this review is not extended to the latter subject.

METHODS AND MATERIALS

Only the final methodology used throughout this work will be given here. Details of research on methodological improvement are described in the first section of 'RESULTS'.

A. Sources and Preparation of Biological Materials.

1. Cells

Here will be described the types and sources of cells used as well as the methods for preparation of each type of cells.

a. Bone Marrow Cells

Bone marrow cells were obtained from the male Royal Victoria Hospital strain rats weighing about 200 gms, in the following manner:

1) Rats were killed by drawing blood from a jugular vein under ether anesthesia.

2) Bone marrow cell suspensions were made from femurs by the following modification of the method of Goodman and Congdon (82). As soon as an animal was exsanguinated, femurs were excised and both ends were cut off. Marrow plugs were forced out with 10 ml of Krebs-Ringer bicarbonate solution (138) (pH=7.4) containing 100 mg% glucose. Cell suspension was made by crushing the marrow plugs in a hand homogenizer.

3) The suspended cells were washed three times with 5 ml of the above solution by alternative centrifugation (for 3 minutes at 900 x g) and resuspension in an International clinical centrifuge.

4) Final cell suspensions were made in 10 ml of the same solution and nucleated cells were counted on a hemocytometer using Turk solution.

5) An aliquot of the cell suspension containing 10^7 nucleated cells was used for each experiment.

b. Spleen Cells

The spleens were obtained from the same strain of rats as used for bone marrow cells. Spleen cell suspensions were prepared in the following manner:

1) Animals (rats) were killed in the same manner as above.

2) The abdomen was opened, and spleen was excised and placed in a beaker containing Krebs-Ringer solution with glucose.

3) It was then minced by a pair of fine scissors into pieces of about 2 mm in diameter.

4) The contents of the beaker were poured onto several layers of surgical gauze which had been placed over another beaker, allowing only dispersed cells to pass through.

5) The suspended cells were washed once and resuspended in 10 ml of Krebs-Ringer solution containing 100 mg% glucose, and nucleated cells counted on a hemocytometer using Turk solution.

6) An aliquot of the cell suspension containing 10^7 nucleated cells was used for each experiment.

c. Peripheral Lymphocytes

Peripheral lymphocytes were prepared according to the method described by Walford et al (283) and modified by Amos (9):

1) 10 ml of venous blood was drawn into a dry syringe and defibrinated in an Erlenmeyer flask containing 20 glass beads (4 mm in diameter) by rotating the flask for 10 minutes. In some experiments, heparinized blood was used, the blood being drawn into a syringe containing heparin (20u per ml of blood).

2) The blood was mixed with 1/4 volume of plasmagel in a test tube, and set at 15^o angle for 10 minutes, then at a 45^o angle for 10 minutes and lastly in a vertical position for 10 minutes to allow red cells to settle.

3) The upper layer was transferred to a centrifuge tube using a Pasteur pipette.

4) By centrifugation for 3 minutes at 900 x g in an International clinical centrifuge the cellular elements were brought down to the bottom of the tube. Most of the supernate was removed leaving 1 ml from the bottom. The cells were then dispersed using a Pasteur pipette.

5) The suspended cells were passed into nylon fibers packed in a Pasteur pipette, which was then kept at 37.5^oC for 18 minutes

to allow polymorphonuclear leukocytes to adhere to the nylon fibers.

6) Lymphocytes and erythrocytes were flushed out with 10 ml of Krebs-Ringer solution and then centrifuged down at 900 x g for 3 minutes. The supernate was discarded.

7) To the cell button, 0.5 ml of supernate from step 4 was added and the cells resuspended.

8) To the cell suspension, 5 drops of Anti-ABO blood group serum were added and the tube shaken horizontally for 5 minutes at room temperature. (Anti-H testing fluid was used for Type O blood.)

9) Agglutinated red cells were removed by slow centrifugation (for 1 minute at 30 x g).

10) The resulting upper layer was transferred to a centrifuge tube and centrifuged for 3 minutes at 900 x g. The supernate was discarded.

11) Precipitated cells (mostly lymphocytes) were washed twice with 5 ml of barbitol buffer.

12) Lymphocytes were adjusted to a count of 2 million per ml in barbitol buffer.

2. Sera or Plasmas

Human donors of serum or plasma were (a) healthy hospital personnel , (b) pregnant women, (c) non-uremic patients who had previously received multiple blood transfusions, (d) uremic patients

without history of blood transfusion, (e) uremic patients who had many blood transfusions and hemodialyses and (f) renal transplant recipients on immunosuppressive medication. Since horses were used for production of anti-human lymphocyte serum, sera were also obtained from normal and immunized horses.

Sera were obtained from clotted blood. Plasmas were obtained from heparinized blood using 20 u of heparin (preserved by benzyl alcohol) per ml of blood. The latter point is stressed here as some degree of anti-cytotoxic action of heparin is to be described later in 'RESULTS'.

In the study of xenogeneic cytotoxicity, human sera and plasmas were used fresh or upon the first thawing after storage at -25° to -65°C for not more than two weeks in order to ensure that antibody-complement system would be at full strength (232). This precaution was necessary as it was undesirable to use exogenous complement (e.g. rabbit or guinea pig serum), which often exerts heterotoxicity and would make difficult the interpretation of the effect of human serum (or plasma).

In contrast, in the study of allogeneic lymphocytotoxicity, human sera were used after freeze-thawing of as often as five times, together with fresh rabbit serum as a source of complement. The horse anti-human lymphocyte serum was also treated the same way.

3. Complement

Rabbit complement was exclusively used in the study of allogeneic lymphocytotoxicity of human sera (283). Sera from three rabbits were pooled, stored at -65°C in small amounts, and used only once after the first thawing. Rabbit serum was used unabsorbed since our preliminary observations confirmed the findings of Amos (7) who reported that the rabbit's natural heterospecific antibody appeared to be directed against human red cells but not against lymphocytes. And it was found by the candidate that polymorphonuclear leukocytes from some human subjects were damaged by rabbit serum, whereas no appreciable damage was exerted by rabbit serum on lymphocytes of any human subject.

B. Techniques for Quantitation of Cytotoxicity.

1. C^{14}-Uridine Incorporation: Liquid Scintillation Counting.

This technique was used to measure functional vitality (in contrast to morphological viability) of cells as judged by the rate of ribonucleic acid synthesis.

a. Incubation of Cells

i. Bone Marrow Cells

1) An aliquot of cell suspension containing 10^7 bone marrow cells was added to a 25 ml Erlenmeyer flask.

2) 0.2 ml of human serum (or 1.0 ml of plasma) was added to the same flask.

3) The volume of the incubation mixture was made up to 3.0 ml by adding Krebs-Ringer bicarbonate solution containing 100 mg% glucose.

4) Finally 0.1 ml of uridine-2-C^{14} solution containing 1μc of radioactivity was added to the mixture.

5) Incubation was carried out for 30 minutes in a Dubnoff metabolic shaking incubator at 37.5°C under an atmosphere of 95% oxygen and 5% carbon dioxide.

ii. Spleen Cells

1) An aliquot of cell suspension containing 10^7 spleen cells was put in a 25 ml Erlenmeyer flask.

2) 0.4 ml of human serum was added to the same flask.

3) The volume of the incubation mixture was made up to 1.5 ml by adding Krebs-Ringer bicarbonate solution containing 100 mg% glucose.

4) Finally 0.1 ml of uridine-2-C^{14} solution containing 1uc of radioactivity was added to the mixture.

5) Incubation was carried out for 60 minutes in a Dubnoff metabolic shaking incubator at 37.5°C under the atmosphere of 95% oxygen and 5% carbon dioxide.

b. Post-incubation Procedure: Extraction of Radioactive Nucleic Acids.

This is, in essence, a modification of the method originally described by Schneider (229). As can be seen below, however, a considerable simplification was made without undue contamination of the final product (C^{14}-nucleic acid) by the precursor (C^{14}-uridine).

1) Incubation of cells was terminated by adding 2 ml of ice-cold saline to the flask.

2) The entire content of the flask was transferred to a 12 ml centrifuge tube. The flask was then washed with 5 ml of ice-cold saline and the washing was added to the tube.

3) The tube was then centrifuged in an International clinical centrifuge at a maximum speed (900 x g) for 3 minutes and the supernate was removed.

4) To the precipitated cell button, 2 ml of sodium lauryl sulfate buffer (0.50 gm of sodium lauryl sulfate, 0.06 gm of tris-hydroxymethyl-aminomethane and 0.58 gm of sodium chloride, dissolved in 100 ml of distilled water) was added and mixed using a Pasteur pipette. This step caused instantaneous lysis of all cells.

5) To this, 3 ml of 20% trichloroacetic acid containing 0.1% non-radioactive uridine was added and mixed as above. The tube was then centrifuged for 10 minutes at 900 x g.

6) The acid-insoluble precipitate was washed once with 2 ml

of 95% ethanol and twice with 3 ml of 10% trichloroacetic acid containing 0.05% non-radioactive uridine.

7) The final acid-insoluble precipitate was dispersed in 10 ml of scintillation solution (0.7% PPO, 0.03% POPOP and 10% napthalene dissolved in dioxane) and transferred into a Packard glass counting vial.

c. Radioactivity Counting.

All radioactivity countings were done in a Packard Tri-Carb liquid scintillation spectrometer using two channels: one, set at a gain of 60% and a discriminator opening of 50 to the infinite, and the other, set at a gain of 9% and a discriminator opening of 50 to 1,000. The counting time was one minute.

d. Grading of Cytotoxicity.

The cytotoxicity was expressed in terms of percentage cell viability using a formula,

$$\frac{\text{radioactivity count in test incubation}}{\text{radioactivity count in control incubation}} \times 100$$

This expression is referred to as V_1 in 'RESULTS'. In fact, this is equivalent to the reciprocal of the cytotoxic index [103].

2. Trypan Blue Staining.

This technique was employed as a means of determining

morphological viability of cells as based on the principle of exclusion of dye by viable cells. Throughout this work, the trypan blue solution was first made 1% in distilled water as a stock solution and appropriate dilutions in saline were made immediately preceding its addition to cell suspension or sedimented cells to give the final dye concentration of 0.1%.

a. Incubation of Cells.

i) Bone marrow and spleen cells

Bone marrow and spleen cells were incubated exactly the same way as described in the preceding section, except for the omission of radioactive uridine.

ii) Peripheral lymphocytes

1) Into each well of a microtiter plate were added 1 drop of human sera, 1 drop of lymphocyte suspension and 1 drop of rabbit complement.

2) The above plate was incubated for 25 minutes at 37.5°C.

b. Staining and Reading of Incubated Cells.

i) Bone marrow and spleen cells

1) A drop (by a Pasteur pipette) of incubated

cell suspension and a drop of 0.2% trypan blue solution were mixed in a test tube and left for a few minutes.

2) A drop of the above mixture was placed on a hemocytometer, then stained and unstained nucleated cells were counted microscopically.

ii) Lymphocytes

1) Following incubation, the plate was placed on crushed ice and the supernate was removed from each well, care being taken not to disturb sedimented cells.

2) 1 drop of 0.12% trypan blue solution was added to each well and the cells were mixed thoroughly using a Pasteur pipette.

3) A drop of the cell suspension was placed on a hemocytometer, then stained and unstained lymphocytes were counted microscopically.

c. Grading of Cytotoxicity.

The cytotoxocity in the dye exclusion technique was expressed in terms of percentage cell death using a formula, "cytotoxicity index (C.I.) x 100", and graded as seen in the following chart. The control indicated percentage cell death in incubation of cells with autologous or allogeneic (normal) plasma or serum.

C.I. * x 100	Grade
Less than 5	0 (negative)
5 - 10	± (doubtful)
10 - 30	+
30 - 60	++
60 - 95	+++
95 - 100	++++

$$* \text{ C.I.} = \frac{\text{\% viable cells in control - \% viable cells in experiment}}{\text{\% viable cells in control}}$$

RESULTS

A. Developmental Research to Improve Methodology.

1. Preparation and Morphology of Cells.

Light microscopic morphology and viability (as judged by trypan blue staining) of cells prepared by various techniques will be described in the following.

a. Bone Marrow Cells

Several techniques have been reported for preparation of bone marrow cell suspension (82, 235, 259). When rat bone marrow cell suspensions were prepared by the original technique of Goodman and Congdon (82) which dispersed cells by flushing the bone marrow plugs through a fine hypodermic needle (26 gauge), it was often found difficult to push out rapidly the entire contents of a syringe.

For the above reason, the technique of crushing the marrow plugs gently in a hand homogenizer was employed. In comparing these two techniques, the viability of suspended cells was same between the two, that is, trypan blue dye stainabilities averaged 13.4% and 13.9%, respectively.

The composition and average yield of cells obtained from each femur of 10 rats by the modified technique were as follows:

No. of nucleated cells = 1.1×10^8

No. of red blood cells = 0.6×10^8

b. Spleen Cells

The original technique described by Perreta et al [206] which employed a hand homogenizer to disperse cells caused a considerably high percentage of cell damage, up to 28.3%. This was presumably due to the strenuous homogenization necessary to crush a firm tissue such as spleen, in contrast to a soft tissue like bone marrow. The latter could easily be crushed by a hand homogenizer.

By slicing the spleen and flushing the dispersed cells through a metal sieve, cell damage was greatly minimized to 16.9%. However, this modification caused a much lower yield of nucleated cells. The comparative features of these two techniques are given below. The figures are averages of values obtained from 6 experiments with each technique.

	Homogenization	Slicing
No. of nucleated cells	4.6×10^8	1.7×10^8
No. of red blood cells	2.7×10^8	2.5×10^8
Trypan blue staining	28.3%	16.9%

c. Peripheral Lymphocytes

In order to devise a most simple and efficient method of isolating lymphocytes from whole blood, the literature on this subject was extensively reviewed and a number of techniques were tried and some modifications were made by the candidate before adopting the

final protocol for lymphocyte preparation as described in 'METHODS AND MATERIALS'.

It is needless to say that isolation of lymphocytes should mean removal of other cellular elements from whole blood, i.e. erythrocytes, granulocytes and platelets. Although the number of papers concerning this problem is very large, there are only a few principles involved in the separation of blood cells as reviewed below.

For removal of erythrocytes, the techniques reported are: (i) flotation methods employing dense chemical agents and making use of differing densities of the different types of blood cells (111, 264, 265), (ii) sedimentation methods using such substances as fibrinogen, dextran, phytohemagglutinin, gelatin, or red cell blood group anti-serum, which increase the erythrocyte sedimentation rate (44, 111, 157, 181, 234, 258), (iii) centrifuge method, merely depending on differing densities of blood cells with or without use of specially designed centrifuge tubes (35, 73, 117) and (iv) hypotonic lysis of erythrocytes based on the differing osmotic resistance of different blood cells (63, 282).

For removal of granulocytes different principles also have been applied: (i) differential centrifugation making use of the differing densities of lymphocytes and granulocytes (73, 117), (ii) use of phagocytic property of granulocytes (36, 132, 153) and (iii) use of

adhesive property of granulocytes (78, 121, 209, 282, 290).

For removal of platelets two different techniques have been used: (i) defibrination of whole blood with glass beads (44, 50) and (ii) use of adhesive property of platelets (78, 282).

The candidate has compared the efficiency of several different techniques for isolation of lymphocytes. These include: (i) one-stage centrifugation (117), (ii) two-stage centrifugation (73, 117), (iii) plasma-gel sedimentation plus nylon column separation (9) and (iv) use of Anti-ABO blood group sera in addition to the last (258).

A brief description of each of the above four methods is as follows:

Method i : 10ml of heparinized blood was centrifuged in a clinical centrifuge at 500 x g for 5 minutes and the supernate, together with the upper layer of buffy coat, was decanted for cells.

Method ii : Following the above centrifugation, the supernate was once more centrifuged at 100 x g for five minutes and the supernate was decanted for cells.

Method iii : 10ml of heparinized or defibrinated blood was mixed with a quarter volume of plasmagel and sedimented for 30 minutes. The supernate was passed through a nylon column in a Pasteur pipette, and the eluate collected for cells.

Method iv : To the above eluate, anti-ABO blood group serum was added and agglutinated red blood cells were removed by

slow centrifugation (30 x g for 1 minute). This is the method used by the candidate in most of the experiments. The detailed description of this method appears in 'METHODS AND MATERIALS'.

From the final cell suspensions, cell counts were performed on a hemocytometer. Lymphocytes and granulocytes were counted after mixing with Turk solution. For erythrocyte count, the cell suspension was used undiluted. In Table I, the efficiency of each technique is expressed according to the following formulas which were originally proposed by Jago (117). The data were obtained using blood samples from healthy individuals.

$$\text{Percentage yield of lymphocytes} = \frac{\text{Volume of final cell suspension x number of lymphocytes per cmm}}{\text{Volume of whole blood x number of lymphocytes per cmm}} \times 100$$

$$\text{Percentage purity of lymphocytes} = \frac{\text{Number of lymphocytes per cmm in final suspension}}{\text{Number of leukocytes per cmm in final suspension}} \times 100$$

$$\text{Percentage of erythrocyte contamination} = \frac{\text{Number of erythrocytes per cmm in final suspension}}{\text{Number of total cells per cmm in final suspension}} \times 100$$

As seen in Table I, Method iv was found most satisfactory in the isolation of pure lymphocytes from whole blood.

Since we are particularly concerned with the partners of cadaver organ transplant (uremics and cadaver donors), a further study was made of the efficiency of Method iv in the isolation of lymphocytes from blood samples of these two groups of subjects. From the first group (potential kidney recipients) blood samples were drawn a few days after the last hemodialysis. From the second group (cadaver kidney donors) blood samples were usually drawn by necessity shortly after or during blood transfusion. The data on lymphocyte preparations from these two groups are shown, along with those from healthy individuals, in Table 2. It will be noted in the table that even with the finest method the isolation of pure lymphocytes is rather difficult in the recently blood transfused individuals. This must be due to a decrease in the adhesive function of granulocytes as well as some loss of agglutinability on the part of erythrocytes.

2. Quantitation of Cytotoxicity.

Many techniques have been reported for examining viability of cells following exposure to various cytotoxic agents. These include (i) dye exclusion technique using trypan blue [89, 104, 108, 182, 200, 220, 246, 283] or eosin [89, 134, 149, 183, 220, 230, 246, 254, 256]; (ii) observation of cell deformity [220]; (iii) phase contrast microscopy [15, 252]; (iv) use of a Coulter counter [256]; (v) measure-

ment of respiratory rate (15, 110, 252) or glycolytic-activity (15, 110, 149); (vi) cultivation of cells in vivo (149, 220, 249) or in vitro (246), (vii) rate of uptake or release by cells of radioactive materials such as C^{14}-glycine (15), C^{14}-thymidine (108, 134), P^{32} (110) and Cr^{51} (225, 226); and (viii) most recently a fluorochromatic assay (21, 37).

With respect to simplicity and sensitivity, each of the above listed techniques has some advantages and disadvantages. For example, the dye exclusion technique was considered relatively subjective and insensitive (246), although this insensitivity is advantageous when cells are tested after a period of storage. On the other hand, the techniques employing release of radioactivity from pre-labelled cells, although sensitive, seem to pose some difficulty in determining the value for 100% cell death which should serve as a basis for quantitation of cytotoxicity.

Since the synthesis of macromolecules is one of the integral functions of cells, it was felt that the rate of incorporation of a radioactive precursor could be used as an index of cell viability under various experimental conditions, provided that the same substance in non-radioactive form is not present in incubation mixture. With this line of reasoning, C^{14}-uridine incorporation was selected, in conjunction with the time-tested trypan blue staining technique, as a parameter to quantitate cytotoxic reactions in this work.

Having chosen the two techniques (C^{14}-uridine incorporation and trypan blue staining) for cytotoxicity study, various trials were carried out to make them more reliable and simple, as described below.

a. C^{14}-Uridine Incorporation

To support the use of this method in measuring cytotoxicity, its reliability and rapidity of performance needed to be substantiated.

First of all, the final precipitate (C^{14}-RNA) to be counted for radioactivity, had to be free from any contamination by its precursor, C^{14}-uridine. This required an effective means of cell disruption and a complete separation of acid-soluble cell components from acid-insoluble ones. The cell disruption was effected with the use of sodium lauryl sulfate ($NaCl_{12}H_{25}SO_4$), an anionic detergent, which was used by Marmur (165) for the isolation of DNA. Subsequent precipitation of acid-insoluble components required relatively high concentration of trichloroacetic acid (TCA) (12%) and longer period of centrifugation (10 minutes). The precipitate was usually rather solid. This solid mass could easily be dispersed with 95% ethanol in which nucleic acids are insoluble, whereas lipids, nucleotides and other smaller molecules are soluble. Following the alcohol treatment, the resulting precipitate was twice washed with 10% TCA containing 0.05% carrier uridine.

To examine the effectiveness of the above procedure in com-

pletely eliminating free radioactive uridine from the final precipitate, two criteria were applied: (1) The final TCA washing should be free of radioactivity. (2) The final TCA precipitate from cells which had been exposed to C^{14}-uridine in an ice bath or incubated at 37°C with non-metabolizable material, e.g., radioactive aminobutyric acid, should contain no radioactivity at all.

The first criterion was met since the radioactivity counts in the last TCA washings from 19 experiments were virtually none, ranging from 0 to 70 c.p.m.

The second criterion was also met as seen in the following two types of experiments:

First, when cells were exposed to C^{14}-uridine in an ice bath, the final TCA precipitates did not contain any radioactivity at all, whereas intermediary washings contained high radioactivity. This is understandable in view of the absence of cellular metabolic activity under these circumstances.

Second, following incubations of cells with alpha-aminoisobutyric-1-C^{14} acid containing 1^{uc} of radioactivity at 37.5°C for 60 minutes, the final TCA precipitates were found to contain no radioactivity. This also proves that separation of acid-soluble cellular components from acid-insoluble was complete.

To assess further the reliability of this technique, a time-course study was made on several occasions. Fig. 1 shows a steady increase in radioactivity with prolonged incubations.

Preparation of the final TCA precipitate for radioactivity counting was also simplified using the suspension technique as described in 'METHODS AND MATERIALS'. This suspension technique was reported by Hayes et al [100], and eliminates a lengthy step involving the use of hydroxide of hyamine [274] to solubilize the acid precipitate. Comparison of radioactivity counts from identical samples using each of these two techniques is given in Table 3a. The efficiency of the former method ranged from 78.0% to 88.0% of the latter. The suspension technique yielded radioactivity counts equivalent (92.5 - 114.0%) to those obtained with the techniques using a filter paper [26, 284] as seen in Table 3b. Furthermore, reproducibility of the suspension technique examined in all the duplicate samples averaged 92.4% (89.5 - 96.0%).

With this suspension technique, an average radioactivity of the final precipitates from incubations of rat bone marrow cells (10^7) with autologous plasmas was 7,040 c.p.m. (4390 - 8390) for 30 minute incubations and 10,870 (5,540 - 17,700) c.p.m. for 60 minute incubations. The corresponding value for rat spleen cells (10^7) was 2,300 (1,670 - 2,880) c.p.m. for 60 minute incubations.

The extent of cytotoxicity of any human serum or plasma was expressed in terms of percentage cell viability, namely, the ratio of radioactivity count from experimental incubation to that from control incubation, as already defined in 'METHODS AND MATERIALS'.

b. Trypan Blue Staining

Since Evans and Schulmann (62) stated that trypan blue diffuses instantly into injured leukocytes, this dye, as well as other vital staining dyes, has been used by many investigators in the study of cytotoxicity phenomenon in various cell lines including normal lymphoid cells and malignant cells.

Opinions differ on the optimal concentration of the dye to be used. Pappenheimer (200) originally used the trypan blue in concentrations of 0.01% and 0.02% to stain rat thymus cells suspended in aqueous fluid and serum, respectively. Gorer and O'Gorman (89) subsequently employed trypan blue at a concentration of 0.05% to examine mouse spleen and tumor cells. Tennant (246), studying toxicity as well as staining efficiency of trypan blue at concentrations of 0.1% to 0.25%, found it non-toxic up to 0.2%. Walford (283) used trypan blue at a concentration of 0.2% in serologic typing of human lymphocytes.

In the experience of the candidate, trypan blue effectively stained injured cells suspended in aqueous solution at a concentration of as low as 0.01%. Lower concentrations were not tried. When cells were suspended in serum-containing medium, trypan blue concentrations lower than 0.04% were sometimes not effective in staining injured cells, depending on the relative amounts of sera present. On the other hand, when trypan blue was used at con-

centrations of above 0.125%, stained cells were difficult to count due to a bluish background. From these observations, it was concluded that the optimum concentration of trypan blue was 0.1%. This concentration was also effective when trypan blue was added to a cell suspension containing as much as 50% of serum. This is somewhat in disagreement with Engelfriet (60) who stated that the optimal concentration of trypan blue was 0.18% for examining cells suspended in serum-containing medium.

With lymphocytes prepared by the technique described in 'METHODS AND MATERIALS', average cell viability in control incubations, as judged by trypan blue dye exclusion, was as follows:

Source of cells	Number of observations	Cell viability (%)
Healthy individuals	11	94.4 ± 0.7*
Uremic patients on periodic hemodialysis	10	84.7 ± 1.7
Cadaver kidney donors following blood transfusions	5	65.1 ± 7.8

* Mean ± standard error

It will be noted that the percentages of cell viability closely corresponded to the percentages of purity of lymphocyte preparations (see the middle row of Table 2). From this, one could suspect that the viable cells are mostly lymphocytes and that the non-viable ones

are contaminant polymorphonuclear cells which must have been damaged before or during incubation. An example of granulocyte damage during incubation is illustrated in the following experiment. A mixture of lymphocytes and granulocytes (3:1) from a healthy person was incubated in three different media: normal human serum, rabbit serum, or normal human serum plus rabbit serum. After incubation, trypan blue was added and cell viability in each incubation medium was examined on a hemocytometer, giving rise to the following result:

Incubation mixture	Stained cells	Unstained lymphocytes	Unstained granulocytes
	%	%	%
Normal human serum and cells	4	64	32
Rabbit serum and cells	30	70	0
Normal human serum, rabbit serum and cells	28	72	0

The above table also implies that unabsorbed rabbit serum is non-toxic to human lymphocytes whereas it can be toxic to human granulocytes.

Trypan blue does not stain injured erythrocytes. This conclusion is based on a microscopic observation of erythrocytes exposed

to hypotonic solution which were slowly undergoing lysis. This unstainability is probably due to the absence of cytoplasmic granules in erythrocytes [62].

c. Correlation of the two techniques: C^{14}-uridine incorporation and trypan blue staining.

In 28 experiments, C^{14}-uridine incorporation and trypan blue staining techniques were used simultaneously in order to determine a correlation between the two with respect to the quantitation of cytotoxicity. In this study, cell viability was expressed in the following three ways:

In the C^{14}-uridine uptake study, the expression $V_1 = \frac{E_1}{C_1} \times 100$ (%) was used, where E_1 = radioactivity count from experimental (test) incubation and C_1 = radioactivity count from control incubation.

In the trypan blue staining study, two expressions were used. First, $V_2 = \frac{E_2}{C_2} \times 100$ (%), where E_2 = percentage of unstained cells in test incubation and C_2 = percentage of unstained cells in control incubation. Second, $V_3 = \frac{E_3}{C_3} \times 100$ (%), where E_3 = number of unstained cells in test incubation and C_3 = number of unstained cells in control incubation. Here, the number of cells refers to that counted in a unit area of a hemocytometer.

Data from the above experiments are shown in Table 4, and

plotted in Fig. 2. Fig. 2 shows that V_1 correlates well with both V_2 and V_3, the correlation coefficients [236] being 0.716 and 0.866, respectively. Both correlations are significant at the level of $P < 0.01$. However, as can be seen in Table 4, individual correlations between V_1 and V_2 in experiments 9, 11, 12, 13, 16 and 21 are quite poor. It should be pointed out that in these instances there was more than 50% decrease in the total number of cells (stained plus unstained), indicating disruption of many cells which should have been counted as stained. This kind of discrepancy is not marked in individual correlations between V_1 and V_3. In other words, V_3 can be used to correct for disappearance of damaged cells due to complete disruption, if precautions are taken against loss of cells due to other than cytolysis.

B. Xenogeneic Cytotoxicity System.

1. Differential Cytotoxicity of Human Plasma or Serum on Rat Cells.

Data presented here show not only a general xenogeneic cytotoxicity of human plasma or serum on rat bone marrow and spleen cells but also different degrees of cytotoxicity (differential cytotoxicity) of plasmas or sera from human subjects with a history of exposure to human tissue antigens as compared to those without

such a history. The latter includes healthy males, healthy nulliparous females and uremic patients who have not had blood transfusion. The former includes multiparous females, multi-transfused persons, chronic uremics on a long-term hemodialysis program and renal allograft recipients on immunosuppressive medication. Some of these data were presented at the 1967 meeting of the Federation of American Society for Experimental Biology (198).

These studies were done mainly using C^{14}-uridine incorporation technique and the results are expressed in terms of percentage cell viability. A clear distinction is maintained between plasma (heparinized) and serum of a given individual, as the degree of cytotoxicity is quite different between the two.

a. On rat bone marrow cells

Fig. 3 shows the varying degrees of cytotoxicity of human plasmas on rat bone marrow cells.

Allogeneic (rat) plasmas exerted a mild degree of inhibiting effect (68.5 - 93.5%) as compared to autologous plasmas.

Xenogeneic cytotoxicity of normal human plasmas was to the level of 36.5% of control for males and 36.2% for nulliparous females. Since these two groups had not been exposed to human tissue antigen, it was felt logical to combine the values obtained from both groups for the purpose of comparison with the values

from other groups listed on the figure. This yields 36.4% as the mean percentage of cell viability in normal human plasma and 10.3% as the standard deviation. Hence, the range of 36.4 ± 20.6% (Mean ± 2 S.D.) as indicated by a vertical bar in Fig. 3, can be regarded to cover 95.5% of all possible percentages of cell viability in normal human plasmas. Accordingly, any values falling below its lower limit (15.8%) can be considered, at 2.5% rarity [164], to indicate the presence of excessive cytotoxicity in any given plasma.

The cytotoxicity of plasmas from uremic patients without previous blood transfusion was in the same magnitude as that of the normal subjects.

Some plasmas from human tissue-exposed subjects were much more toxic than those from normal subjects, showing cell viability of less than 15.8% of control. This portion of toxicity, in excess of natural xenogeneic toxicity, is interpreted to represent 'heterophile cytotoxicity' in this thesis.

The role of dialyzable uremic toxins was also studied using rat bone marrow cells. Two types of experiments were done: (i) incubation of cells with plasmas from the same uremic patients before and after a period of six hours of hemodialysis and (ii) incubation of cells with pre- and post-dialysis bath solutions. In the latter, the bath waters were ultrafiltered to eliminate microbial contamination. Fig. 4 shows that there was no increase in viability of cells when

incubated with post-dialysis plasmas compared to those incubated in pre-dialysis plasmas. Also, there was no decrease in cell viability when post-dialysis bath solutions were compared to pre-dialysis bath solutions as might be anticipated if dialyzable uremic toxins were of importance in cytotoxicity. It is therefore concluded that dialyzable uremic toxins do not exert any damage to rat bone marrow cells under these experimental conditions.

Xenogeneic toxicity on rat bone marrow cells was also studied using human sera (instead of plasmas) by the same technique. These sera were also tested for allogeneic (anti-human) lymphocytotoxicity by trypan blue staining technique and a relation was sought between xenogeneic and allogeneic cytotoxicities of individual sera.

Fig. 5 shows a general pattern of differential cytotoxicity of human sera similar to that of human plasmas.

Xenogeneic cytotoxicity of normal males' sera and that of nulliparous females' sera were to the level of 28.9% and 27.8% of control, respectively. Considering these two groups together as unsensitized subjects, the mean and standard deviation of percentages of cell viability were 28.4% and 8.2%, respectively. Thus, 95.5% of all possible values for normal human sera should fall within the range of $28.4 \pm 16.4\%$ (Mean $\pm$ 2 S.D.), the lower limit being 12.0%.

From Fig. 5, it is again evident that sera from some of the humans exposed to human tissue antigens exerted stronger toxicity

on rat bone marrow cells when compared to normal human sera.

In the allogeneic system, none of the sera from normal and uremic (non-hemodialyzed) subjects showed lymphocytotoxicity.

It will be noted, however, that sera from multiparous women can be divided into four different groups: (i) those with neither heterophile cytotoxicity nor allogeneic lymphocytotoxicity, (ii) those with heterophile cytotoxicity only, (iii) those with allogeneic lymphocytotoxicity only and (iv) those with both. On the other hand, sera from male or nulliparous female uremics on repeated hemodialysis fall into two groups only: (i) those with neither heterophile nor allogeneic cytotoxicity and (ii) those with both.

Among sera from long surviving renal allograft recipients, all of whom had had repeated hemodialyses prior to transplantation, some showed definite heterophile cytotoxicity but none demonstrated allogeneic lymphocytotoxicity. As all of the allograft recipients were receiving immunosuppressive drugs, one cannot rule out the possibility that lymphocytotoxicity could be detected in these sera before the initiation of immunosuppressive regimen.

b. On rat spleen cells

Although a number of experiments were performed earlier, using C^{14}-uridine, to study the effect of human plasmas on rat spleen cell activity, the data from these experiments were not conclusive

for the following reason. In these instances, spleen cells were incubated in the number of 10^8. This number was probably too large so that either the cell excess masked the result of death of a portion of cells due to cytotoxins, or the emission of radioactivity was hindered by excessive mass of the final precipitate.

In later experiments, cytotoxicity of human sera on rat spleen cells was measured using only 10^7 cells as described in 'METHODS AND MATERIALS'. Fig. 6 shows these results expressed as percentage cell viability. Human sera are evidently far more toxic to rat spleen cells than allogeneic (rat) sera which have only a low degree of inhibitory effect.

Although it is apparent in Figure 6 that some sera from multiparous women were more toxic than normal human sera, a statistical analysis was not attempted because of insufficient number of experiments with normal sera. It is, however, again noted that there was no correlation between xenogeneic and allogeneic cytotoxicities in sera from multiparous women.

2. Factors Modifying Xenogeneic and Heterophile Cytotoxicities of Human Plasmas on Rat Cells.

The purpose of the experiments described here is to characterize the mechanisms or factors responsible for the induced heterophile cytotoxicity, as well as for the natural xenogeneic cytotoxicity,

Various means were used to modify human plasmas, including those described by Landry et al [149].

a. Ultrafiltration of plasma

The first step was to localize cytotoxic factors in one of two portions of plasma: ultrafiltrable and non-ultrafiltrable.

Whole plasmas were ultrafiltered by the method of Nicholas [197]. Filtrates and original whole plasmas were used separately to incubate cells under identical conditions, and cell viability observed. In the left half of Fig. 7, values for cell viability obtained with normal and sensitized human plasmas are expressed as percent of the value obtained with autologous plasmas. In the right half of the figure are values obtained with ultrafiltrates of plasmas expressed in the same manner. Three whole plasmas and their corresponding ultrafiltrates were studied from three categories of donors: rats (cell donor), normal humans and sensitized humans.

As indicated by the dotted line, the xenogeneic cytotoxicity of normal human plasmas was completely absent from their corresponding filtrates. Similarly, ultrafiltration removed profound toxicity of hemodialyzed uremic patients' plasmas as indicated by the dashed line. These findings imply that factors responsible for both xenogeneic and heterophile cytotoxicities reside in non-ultrafiltrable macromolecules.

b. Heating of plasma

In order to examine thermolability of the cytotoxic factors, human plasmas were heated at 56°C for 30 minutes. The fresh plasmas and their corresponding heated counterparts were separately added to cell incubation mixtures under identical conditions.

The results are presented in Fig. 8. The orientation of the figure is the same as the previous one, there being three experiments with each category of plasma.

The dashed line indicates an almost complete loss of cytotoxicity from plasmas of hemodialyzed uremic patients by heating. Similar loss of toxicity occurred in normal human plasmas, as indicated by the dotted line. This would indicate the thermolabile nature of both xenogeneic and heterophile cytotoxicity systems.

Although heating of plasma causes inactivation of complement as first reported by Ecker et al [57], and is now known to specifically inactivate C'1, C'2, C'5, C'8 and C'9 [193], this does not exclude a possible destruction of other factors which might be responsible for the cytotoxicities in question.

c. Addition of a chelating agent, Ethylenediamine Tetracetic Acid (EDTA).

In order to examine divalent cation dependency of the cytotoxicity systems, a chelating agent, EDTA, was added to the

incubation mixture to give the final concentration of 0.005 M [149]. Three experiments with each category of plasma were done and the results are shown in Fig. 9. As noted in the right half of the figure, the cytotoxicity of plasmas from multi-transfused persons was largely eliminated by the addition of EDTA. This was true, also, of normal human plasmas. The low function of cells does not mean the persistence of cytotoxicity, but is due to the effect of EDTA per se as seen for autologous plasmas plus EDTA. In other words, this is an effect of the depletion of divalent cations which are required for cellular metabolic activities including ribonucleic acid synthesis. In fact, the cells incubated in EDTA-containing mixtures were not stainable with trypan blue. The two parameters of cell viability, C^{14}-uridine incorporation and trypan blue staining, did not correlate with each other in this instance.

d. Removal of properdin

By means of the aforementioned three types of experiments, it could be stated that the factors responsible for both cytotoxic reactions, xenogeneic and heterophile, are non-ultrafiltrable, thermolabile and divalent cation dependent. This much characterization does not, however, exclude the possible role of properdin [207, 208, 286], a normal serum protein involved in natural immunity.

Properdin was removed from plasmas in the following manner

by the method of Pillemer et al [207]. 3 mgs. of zymosan was added to 1 ml of plasma at 15°C to 18°C and the mixture was shaken periodically for 75 minutes. It was then centrifuged at 900 x g for 10 minutes at 4°C. The properdin-zymosan complex was precipitated and the supernate lacking properdin (RP) was obtained.

Three plasmas and their corresponding RPs from each category of subjects were tested for cytotoxicity, as in the previous three types of experiments. The results are plotted in Fig. 10. It is evident that removal of properdin from plasmas did not alter the original cytotoxicity; hence, the properdin system is not involved in these phenomena.

All the above evidences imply that both natural and heterophile cytotoxicities are due to antigen-antibody interaction.

e. Selective removal of C'3 or C'4

In an effort to further identify the cytotoxicity systems in question, human plasmas were made selectively free of C'3 or C'4 activity, the partially decomplemented plasma being called R3 or R4, respectively.

R3 was made, according to the method of Pillemer et al [57, 207], by incubating 3 mgs of zymosan and 1 ml of plasma at 37.0°C for 1 hour and separating the supernate from the precipitate by centrifugation. R4 was also made by the method of Pillemer

(207), in which 1 ml of plasma was treated with 0.1 ml of 0.16 M hydrazine and the mixture incubated for 1 hour at 37.0°C.

Three plasmas from each category of subject and their corresponding R3, R4 and R3 + R4 were separately tested with cells under identical conditions. The results are shown in Fig. 11.

First, it can be noted that R3 and R4 from both classes of human subjects were completely devoid of cytotoxicity when compared to autologous R3 and R4. Second, as seen in the far right part of the figure, when R3 and R4 were combined so that all the components of complement were present in the mixtures, most of the original toxicity was restored in both classes of human plasmas. This establishes that these cytotoxic phenomena are complement-dependent.

Data obtained from all of the foregoing experiments show, conclusively, that the heterophile cytotoxicity, as well as the xenogeneic cytotoxicity (149), is dependent on antibody-complement system.

f. Heparin and Protamine

Anti-complementary action of heparin was first reported by Ecker et al (56) and subsequently confirmed by others (93, 135, 202, 245, 293, 294). Heparin was tested also in the present study for its effect, quantitative as well as qualitative, on the cytotoxic reactions in question.

Table 5 shows the anti-cytotoxic action of heparin present

at varying concentrations in incubation medium, and reveals a definite dose-response relation. In general, the more toxic the sera, the more heparin is needed to obtain an equivalent cell viability.

The effect of heparin antagonist, protamine, was also tested in the same cytotoxicity system. As seen in the third column of Fig. 12, protamine also showed anti-cytotoxic effect equivalent to that of heparin. These percentage cell viabilities were confirmed by trypan blue staining. No attempt has yet been made to analyze the mode of anti-cytotoxic action of protamine. The effect of a mixture of heparin and protamine on cytotoxic reaction was not constant, showing a certain evidence of mutual neutralization, as reflected in some restoration of plasma toxicity in one out of three instances.

3. Cytotoxicity of Horse Anti-human Lymphocyte Serum on Human Lymphocytes.

This study was conducted in view of recent reports on the therapeutic value of horse anti-human lymphocyte serum (ALS) in human renal allograft recipients (238). The horse anti-human lymphocyte sera which were tested had been produced by the transplantation research team of the Royal Victoria Hospital according to the method of Iwasaki et al (116), using human thymus tissue obtained at pediatric heart surgery as antigen for immunization of horses.

Although there has been no study yet to correlate in vitro lymphocytotoxicity with in vivo graft survival, it was felt that lymphocytotoxicity test, at least, could serve as an index of effective immunization of horses. Seven different batches of horse ALS preparations were tested against lymphocytes from 10 random individuals, the number of total combinations being 70.

Table 6 shows the results which can be interpreted as revealing the following facts: (i) some ALS preparations are strongly reactive with lymphocytes from all individuals tested and (ii) some ALS preparations react selectively with only a portion of individuals tested and the strength of individual reactions is somewhat lower than the first group of ALS.

C. Allogeneic Lymphocytotoxicity System: Human Serum on Human Lymphocytes.

This section deals with interaction between human serum and human lymphocytes. No plasma was used in this study. The technique of cell incubation has been described in 'METHODS AND MATERIALS'. All reactions were examined by trypan blue staining technique, although at times C^{14}-uridine incorporation was measured in conjunction with the dye exclusion.

Data presented here mainly concern the incidence of lymphocytotoxic antibodies in various categories of human subjects, the

significance of their presence in transplant recipient's serum and their applicability to histocompatibility testing.

1. Screening of Human Sera for the Presence of Cytotoxic Antibodies.

In screening any given serum for the presence of cytotoxic antibodies, the first thing which should be determined is what degree of cytotoxicity can be termed "positive". Walford (282), using trypan blue dye, drew a line at a level of 20% staining as a positive reaction, less than 10% and intermediate values (10-20%) being regarded as negative and equivocal, respectively. Terasaki (252), in his micro-droplet technique, considers 50% cytotoxicity as an indication of a need for further dilution of a serum.

To the candidate, using trypan blue staining, 10% cell death could be regarded significant of a positive cytotoxic reaction, 5-10% being doubtful. This statement is based on the finding that there was no instance in which difference in cell death between duplicates was over 10% in a total of 200 observations, a difference of 5-10% being seen in only 16 instances.

To screen each serum, a panel of 10 random cell donors was used. The minimum requirement to be classed as "positive" was either a positive reaction with lymphocytes from one donor at a level of 20% or more cell death, or a positive reaction with 2 donors at a level of 10% or more cell death.

Further efforts for simplification of the screening procedure resulted in two useful variations: (i) reduction of the size of cell panel from 10 to 5, and (ii) what might be called "mixed cell incubation":

(i) The validity of the reduction of the panel size is based on the fact that when 190 pregnant women's sera were tested with 10 random donor's cells, 90% of the eventual total of 41 positive sera were detectable within the first five tests. Therefore, for the practical purpose, a panel of 5 cell donors seems to be sufficient to detect "positive" sera when screening multipara's sera.

(ii) The technique of so-called "mixed cell incubation" was designed to further facilitate the screening procedure. Theoretically, when a mixture of equal number of 5 different individual's lymphocytes is incubated with a single serum, the result should be at least a 10% cell death if the serum reacts with 50% or more of one of 5 populations in the cell mixture. And the reaction should be negative in the absence of a positive combination. The latter situation is well illustrated in Table 7a which shows the reaction profile of a negative serum tested on a mixture of 5 individuals' lymphocytes as well as on each individual's cells separately.

A mixture of 3 individual's lymphocytes instead of 5 individuals, can be used to observe a higher percentage of cell death if there is one or more positive combinations of serum and cell. This is

illustrated in Table 7b which shows the reaction profile of two positive sera tested on lymphocytes from 3 individuals.

Although the "mixed cell incubation" does not disclose a positive reaction of a serum with selective members of the mixture, this non-selectivity does not invalidate the test inasmuch as it is designed to screen any given serum just for the presence of antibodies. The above simplifications were, however, developed relatively late in the course of this work, and all the data presented in the following sections were obtained by testing each serum on lymphocytes from 10 random individuals separately.

2. Incidence of Lymphocytotoxic Antibodies in Human.

a. Normal, nulliparous humans.

Among more than 50 human sera of the above category which were tested in comparison with autologous sera, there was no instance in which cells incubated with allogeneic human serum showed more than 5% staining above those incubated with autologous serum, the former being very ofter lower than the latter.

The above finding is in complete agreement with that of Terasaki (252), and leads to a conclusion that normal, unsensitized individuals do not contain allogeneic lymphocytotoxic antibodies detectable in vitro. Thus, any normal human serum can be used as control in the lymphocytotoxicity test.

b. Pregnancy

The role of pregnancy in the formation of antibodies to leukocytes in general have been reported since 1958 (205, 269, 273). Later, Terasaki demonstrated human lymphocytotoxins in sera of multiparous women (257) and reported that the incidence of lymphocytotoxic antibodies was 49% among women with 5 or more pregnancies and 16% among those with 1 to 4 pregnancies (252).

A similar analysis of 190 pregnant women attending the maternity clinic at the Royal Victoria Hospital reveals that the values were 43% and 17%, respectively. A more detailed analysis of this series of pregnant women is shown in Table 8. In sampling these pregnant women, except for the groups of the first or second pregnancy, no attempt was made to select the subjects with respect to their parity. It is evident in the table, that there is an inverse relationship between the number of subject and the degree of parity, reflecting a correct sampling of the population.

Although the number of subjects for each parity is not equal, it is clear that the greater the number of pregnancy, the higher the incidence of lymphocytotoxic antibodies. However, an apparent levelling-off of the incidence toward the 5th pregnancy and its abrupt rise at the 6th pregnancy, as noted in the table, cannot be explained adequately.

c. Hemodialysis (pre-transplant)

Terasaki et al [254] reported that the incidence of lymphocytotoxins in patients on a pre-transplant hemodialysis program was 23.9% for the male and 46.25% for the female. The female group, however, included those with a history of pregnancy.

A similar survey was made of a total of 39 pre-transplant uremic patients dialysed at several Canadian hospitals. The duration of hemodialysis ranged from 3 to 13 months. The patients were divided into 3 groups: males, nulliparous females and parous females.

As shown in Table 9, the incidences of lymphocytotoxins in these groups were 24.1% (7/29), 20.0% (1/5) and 40.0% (2/5, respectively.

d. Post-transplant Period

Sera from 33 renal allograft recipients were tested for the presence of lymphocytotoxic antibodies. These patients were studied 3 to 36 months after transplantation, at which time the grafted kidneys were functioning adequately under immunosuppressive measurements. There were 24 males, 6 nulliparous females and 3 parous females, all of whom had been hemodialysed for a period of 2 to 30 months prior to the transplantation.

As seen in Table 10, none of the sera from these renal allograft recipients was found to contain lymphocytotoxic antibodies.

e. Auto-immune disease: Systemic lupus erythematosus (SLE).

van Loghem et al [266] reported the demonstration of both complete and incomplete white cell auto-antibodies in patients with SLE. Antinuclear antibodies against lymphocytes were also demonstrated in the sera of SLE patients [178, 190]. Therefore, an attempt was made by the candidate to detect lymphocytotoxins, autologous or allogeneic, in the same disease state.

Sera from 5 female SLE patients, including one with three pregnancies and one with previous hemodialysis, were tested against lymphocytes from a panel of 10 random donors as well as autologous lymphocytes. In this small number of trials, no lymphocytotoxins, either autologous or allogeneic, were demonstrated.

3. Factors Modifying Lymphocytotoxic Reaction in Vitro.

As described earlier, several factors modifying cytotoxic reaction were examined in the xenogeneic cytotoxicity system. In the allogeneic lymphocytotoxicity system, a few additional factors were tested, as well as some of the factors examined with the former system.

a. Aging of serum; Two-stage incubation technique to reverse the effect of aging.

In the course of studies on anti-lymphocyte sera from multiparous women, it was noted that some of the strong sera became non-reactive after several months of storage at -20°C. This loss of activity could not be explained on the basis of inactivation of complement, since fresh rabbit complement was used in every experiment. To determine whether antibodies were completely destroyed during storage, a so-called two-stage incubation technique was employed, as originally reported by Amos (11) and described in detail by Terasaki et al (250) and Ferrone et al (69).

Table 11 shows data obtained from an illustrative case in which lymphocytes were incubated first with an aged antiserum, then the cells washed and incubated with fresh rabbit serum. By this two-stage incubation technique a possible anti-complementary effect of the aged serum was eliminated and its original toxicity was largely restored.

b. Hydrocortisone

Hydrocortisone is, at present, one of the major immunosuppressive agents used in the management of post-transplant patients. The effect of hydrocortisone on immune reactions in vivo and in vitro has been reported variously.

In experimental animals, hydrocortisone was found to delay

the rejection of allografts [19, 20, 172, 261]. Later, some workers reported that hydrocortisone had no effect on immune lysis in vitro [115, 151, 287]. However, Rosenau et al [222] demonstrated an inhibitory action of hydrocortisone on cellular immunity in vitro. More recently, Fell and Weiss [68], and Jennings [120] have shown a similar effect of hydrocortisone on immune cytolysis by humoral antibody and complement in vitro. As the test system, the former [68] used rabbit anti-mouse serum, and the latter [120], rabbit anti-sheep hemolysin.

In the present work, the effect of hydrocortisone on human lymphocytotoxic reaction in vitro was studied by adding 0.01 ml of various concentrations of Hydrocortisone Sodium Succinate (Upjohn) to a 0.9 ml incubation mixture containing lymphocytes, serum and complement to give desired concentrations of hydrocortisone. The commercially available injectable form of hydrocortisone contains 4 different kinds of additives including preservatives. These additives were found to cause death of lymphocytes at the concentrations in which hydrocortisone per se is protective of cells against immune lysis. These points are shown all together in Table 12.

It will be noted in the table that hydrocortisone definitely inhibits lymphocytotoxic reaction in vitro at very high concentrations: 20 mg/ml or more. The steroid itself does not injure the lympho-

cytes but the additives contained in the commercial product do cause damage to the cells at high concentrations.

c. Heparin and Protamine

As in the study on cytotoxicity of human plasmas on rat cells, heparin and protamine were also tested in the allogeneic lymphocytotoxicity system.

Table 13 shows that heparin and protamine, singly or in combination, is not toxic to cells. Heparin, however, inhibited cytotoxic action of antisera, the inhibition being almost complete at a concentration of 7 mg per ml. Protamine also seemed to cause a slight degree of inhibition of cytotoxic reaction at a concentration of 0.7 mg per ml. Higher concentrations of protamine, however, were not tested due to unavailability of an appropriate preparation. Analysis of effect of a mixture of the two agents is not conclusive from the data presented.

4. Preformed Lymphocytotoxic Antibodies and Immediate Renal Allograft Rejection:

The role of circulating antibodies in the rejection of allografts has been demonstrated in mice by Gorer et al [83, 85], in guinea pigs by Voison et al [277] and in men by Kissmeyer-Nielsen et al [133]. Terasaki et al [251] described the finding of preformed lymphocytotoxic antibodies,

directed against the donor's lymphocytes, in a human kidney allograft recipient. He [254] later reported a similar finding in a series of 7 kidney allograft pairs.

The renal transplant unit at the Royal Victoria Hospital was probably the first to apply the above investigators' findings to the prospective matching of kidney transplant pairs, particularly in a cadaver transplant program.

Dossetor et al [54] reported that in their series of 59 cadaver kidney transplants, the incidence of non-functioning kidney was about 20%. Less than one-half could be attributed to technical problems or excessive ischemic time. Most of the remainder can probably be regarded as a rejection due to preformed cytotoxic antibodies which cause acute injury to vascular endothelium resulting in fibrinoid necrosis of arterial and anteriolar walls (Figs. 13 & 14). This conclusion is based on three phases of experience, as shown in Table 14:

(i) Prior to September, 1967, two patients (I.A. and D.D.) who had previously rejected their allograft immediately, were subsequently shown to contain circulating lymphocytotoxins.

(ii) From September to December, 1967, a direct "crossmatch" test of kidney recipient's serum with donor's lymphocytes was carried out simultaneously with each kidney transplantation. On three occasions (G.R., J.C., and D.D.) where the test was positive, the kidney failed to function, whereas on five occasions (C.Mc., J.W.,

G.W., Y.L., and R.B.) where the test was negative adequate kidney function was maintained at least for a period of time.

(iii) From December, 1967, the direct "crossmatch" test has been done prior to every instance of renal transplantation in order to avoid the immediate catastrophe. Until February 1968, there were three patients who were found to contain a wide-spectrum of lymphocytotoxins but were successfully transplanted with kidney from the corresponding donors on the basis of a negative crossmatch. All these grafted kidneys assumed function post-operatively. Two of these patients (I.A., and D.D.) had previously suffered immediate rejection, once in one (I.A.) and twice in the other (D.D.). Both of them have been maintaining adequate function of their present allograft [55].

A most interesting thing is that the serum of I.A. has been reactive with lymphocytes from 119 out of 120 donors tested, the only negative one (her brother) being used as the kidney donor.

5. Specification of Human Anti-lymphocyte Sera Using a Panel of Pre-typed Lymphocytes:

Specification and classification of antisera have been a major concern of many investigators in the field of histocompatibility. Three methods of analysis have been reported and found useful: (i) chi-square analysis [49, 271], (ii) factor analysis [255] and

(iii) posterior probability analysis [177]. Mickey et al [176] compared these three methods of analysis and discussed their relative merits.

At the Royal Victoria Hospital, through the screening procedure mentioned earlier, 41 antisera were secured from 150 multiparous women's sera tested. In addition to these, 10 more antisera were obtained from the U.S. National Institute of Health. These were originally submitted by Dr. D.B. Amos at Duke University.

In characterizing the above 51 sera as to their specificity, the candidate attempted the following type of retrospective analysis. This was done by testing each antiserum on a panel of lymphocytes from 20 to 50 individuals, all of which were independently typed by Dr. P.I. Terasaki at the University of California in Los Angeles with respect to his 7 major lymphocyte antigen groups [255].

Analysis of individual reactions of antiserum (specificity unknown) with lymphocytes (antigen profile known) yielded the reactivity of each antiserum with respect to each of the 7 major antigen groups, as shown in Table 15.

In interpreting the fraction figures in the table it should be understood that since each individual's lymphocytes contain more than one antigen group, each antiserum may or may not be shown to be reactive with respect to any particular antigen group, depending on some other antigen group(s) present in the lymphocytes and

specific antibodies contained in the serum. For example, antiserum RVH-39 reacted positively with 4 individuals' lymphocytes which contained antigen 2 but negative with 3 other individuals' lymphocytes which also contained antigen 2. The most rational explanation for this discrepancy is that the positive reactions of antiserum RVH-39 with the first 4 individuals' lymphocytes were due to the presence in those lymphocytes of other antigen(s) which the antiserum could recognize. In other words, antiserum RVH-39 most likely does not contain anti-2 antibody. On the other hand, the same antiserum reacted positively with lymphocytes from all of 11 individuals who contain antigen 3. This means that the antiserum most probably contains anti-3 antibody.

A number of combinations of antiserum and antigen showed high percentages of positive reaction. In order to assess the reaction patterns of individual combinations, the following tentative criteria were set up:

Probably positive combination = (a) Positive reaction of antiserum with over 90% of a panel of 10 or more cell donors who contain the particular antigen, or (b) positive with all members of a panel of 5 to 9 cell donors who contain the antigen.

Questionably positive combination = (a) Positive reaction of antiserum with 75 to 90% of a panel of 10 or more cell donors who contain the particular antigen, or (b) positive with over 80% of a panel of 5 to 9 cell donors who contain the antigen.

On the basis of the above criteria, an antibody profile of some of the antisera was determined as shown in the last two columns of Table 15. As noted in the table, one-third of the Royal Victoria Hospital antisera and 8 out of the 10 Duke University's antisera could be specified this way. These specified antisera are currently in use at the Royal Victoria Hospital for typing of lymphocytes from prospective donors and recipients of renal allografts.

Although the reactivity of those unspecifiable antisera was found to be relatively weak compared to those specifiable ones, it is still possible that some of the former group may contain antibodies against some antigens other than the seven already defined. In fact, Dr. P.I. Terasaki has recently been reporting a few new lymphocyte antigen groups. Therefore, it is likely that some of our hitherto unspecifiable antisera will be specified against the newly defined antigens in the near future.

DISCUSSION ON METHODS AND RESULTS

1. Selection of Animal Cell Types for Cytotoxicity Study.

The reasons for selecting bone marrow cells in the study of cytotoxicity were three-fold. Firstly, bone marrow should be one of the metabolically most active tissues in the body inasmuch as it consists of nucleated precursor cells of mature blood elements. This point has been amply illustrated by the rate of its nucleic acid synthesis (118, 160, 166, 206, 235, 260, 262, 263). Antigenicity of bone marrow cells has also been reported (28). Therefore, cytotoxicity as reflected by a decrease in nucleic acid synthesis by bone marrow cells could be measured in a relatively short period of time.

Secondly, the very nature of composition of bone marrow cells, namely, the presence of nucleated erythroid cells and megakaryocytes, besides myeloid cells, could make any effect on these cells measurable by both dye exclusion and nucleic acid synthesis, neither of which could be applied to the study of viability of mature erythrocytes and platelets (14) which do not contain nucleus or cytoplasmic granules.

Thirdly, bone marrow cells are very easy to obtain in suspension form, allowing distribution of equal amounts of cells into the desired number of incubation. In this regard, bone marrow has an advantage over the intestinal mucosa which is also metabolic-

ally active [203, 242] but not subject to quantitation except by the weight. Although some of the preliminary work was done by the candidate using rat intestinal mucosa, its use was discontinued for the above reason.

Spleen was used in this study mainly because of the ease with which a large number of lymphoid cells could be obtained. Spleen cells were also shown to incorporate radioactive precursors into nucleic acids in vitro [64].

2. Isolation of Lymphocytes from Whole Blood.

The candidate tried various techniques of isolation of lymphocytes from whole blood in order to devise a more efficient and easily applicable method (Table I, Method iv). It should be pointed out, however, that all methods reported in the literature are applicable to only fresh blood samples. Data (Table 2) concerning isolation of lymphocytes from recently blood-transfused or hemodialysed persons should be of particular interest to those engaged in serologic tissue typing. As mentioned in 'RESULTS', lymphocyte isolation from these subjects, even with the best of 4 methods tried, is not satisfactory.

3. Use of C^{14}-Uridine for Cytotoxicity Measurement.

C^{14}-uridine is shown to be incorporated into nucleic acids, largely into ribonucleic acid and slightly into desoxyribonucleic acid, in mammalian cells [195, 302]. Since free uridine or its structural analogues are not known to be present in plasma or serum, there is

no dilution factor in the incubation medium which would invalidate the use of radioactive uridine incorporation as a parameter of cell viability.

In the study of the metabolic rate of radioactive materials, separation of radioactive precursor from radioactive end-product should be complete if the rate of synthesis of the latter is to be determined accurately. The demonstrations that radioactivity was absent from the final acid washings after C^{14}-uridine incubation and that the acid precipitate of cells incubated with radioactive aminobutyric acid (non-metabolizable) did not contain any radioactivity, show that this separation was complete.

The suspension technique greatly simplifies the preparation of materials for radioactivity counting. And the reproducibility, in terms of duplicate determinations, was over 90%. Although this method yields lower recovery of radioactivity count compared with the commonly used technique of hyamine solubilization (Table 30), this is not disadvantageous for quantitation of cell viability inasmuch as the viability calculation is based on comparison with control.

The time course study (Fig. 1) showing a steady increase in the rate of C^{14}-RNA synthesis with prolonged incubations of bone marrow cells constitutes further evidence for reliability of the technique.

As shown in Table 4 and Fig. 2, cell viability as determined by C^{14}-uridine incorporation, although it did not correlate precisely

with that judged by trypan blue staining, was quantitatively more accurate than the dye exclusion technique as the latter does not reveal a portion of injured cells which have been disrupted and therefore cannot be counted as stained.

4. Use of Trypan Blue for Cytotoxicity Measurement.

In this work, trypan blue was used in conjunction with C^{14}-uridine for the cytotoxicity study on rat bone marrow and spleen cells and as a sole method for the study of human lymphocytotoxicity. The optimal concentration of trypan blue for cells either in aqueous or in protein-containing medium was found to be 0.1%, although other investigators reported somewhat differently (60, 283).

Although trypan blue staining technique has been reported to be relatively less sensitive in determining cell viability compared with such techniques as metabolic studies, which the candidate has also shown to be true (Table 4), there seem to be a few advantages to this technique. First, since the trypan blue stains only structurally disintegrated cells, regardless of the functional integrity, this dye is useful when cells have to be examined after a period of storage or under some other unfavorable conditions. Second, it is probably more useful than metabolic studies in examining such cells as human peripheral lymphocytes, which are not metabolically very active and may not be readily obtainable in large number. In fact, when C^{14}-uridine incorporation rate in human lymphocytes was

measured, 50 ml of whole blood had to be used and the final acid precipitates had to be counted for radioactivity at least for 5 minutes in order to obtain a radioactivity count comparable to that obtained from 10^7 bone marrow cells. From a few experiments in which the rate of C^{14}-uridine incorporation was compared between bone marrow cells, lymph node cells, spleen cells and peripheral lymphocytes, the ratio was approximately 6:4:1:1.

Thus, trypan blue staining seems to be still one of the most useful techniques for the determination of cytotoxicity, at least, in the system using peripheral lymphocytes.

5. Lymphocytoxicity versus Granulocytotoxicity.

As described in 'METHODS AND MATERIALS', each incubation mixture in lymphocytotoxicity test consists of serum, complement (rabbit serum) and cell suspension added in equal volumes. From a number of such incubations using autologous or allogeneic human serum, the following points are worth mentioning. First, human lymphocytes are not damaged by allogeneic serum or rabbit serum. Second, granulocytes from many human subjects are damaged by rabbit serum. Third, human erythrocytes are very often found to be lysed by rabbit serum.

From the above observations, it seems apparent that the granulocytes are not a reliable indicator of cytotoxicity in the incubation system using xenogeneic (rabbit) serum as complement. If one can use only fresh human serum so that animal serum is not needed

for complementation of the system, the cytotoxicity test may be applicable to granulocytes as well [60].

Erythrocytes were found to be unstainable by trypan blue either in crenated form or during actual disruption by means of hypo-osmotic lysis.

6. Xenogeneic (anti-rat) Cytotoxicity System.

The experiments concerning the interaction of human plasma or serum with rat bone marrow or spleen cells (Figs. 3, 5 and 6) evidently show two phenomena; (i) mere xenogeneic cytotoxicity of human plasma or serum in general and (ii) excessive cytotoxicity of plasma or serum from some of the human subjects who have been exposed to human tissue antigens by means of pregnancy, blood transfusion (including hemodialysis) or possibly renal allograft.

A point of importance is that plasmas or sera from uremic patients without history of blood transfusion did not show any more toxicity than the natural xenogeneic toxicity. As a matter of fact, the first thing looked for using C^{14}-uridine in this work was a possible effect on cellular metabolic rate of uremic toxins contained in plasmas of patients on a long-term hemodialysis program. The data shown in Fig. 4 obtained from the experiments using pre- and post-dialysis uremic plasmas and pre- and post-dialysis bath solutions clearly demonstrate that dializable uremic toxins are not cytotoxic during a short period of 30 to 60 minutes of incubation.

Although the C^{14}-uridine incorporation technique might not be sensitive enough to detect any effect of uremic toxins under these experimental conditions, it was found to be a quite sensitive and quantitative indicator of "immune cytolysis", leading to its extensive use in the study of xenogeneic cytotoxicity reported in this thesis.

The fact that xenogeneic cytotoxicity of mammalian plasma or serum is dependent on antibody-complement system seems to be established beyond doubt by the studies of many investigators, notably Landy et al [149] and Terasaki et al [249], and also by the experiments described in this thesis.

Although most of the candidate's studies on various factors modifying cytotoxic reaction are not original, these factors were tested with a hope of elucidating the nature of the excessive cytotoxicity (heterophile cytotoxicity) exerted by sera from some of the humans exposed to human tissue antigens.

A close analysis of the data obtained from these experiments seems to reveal several points of interest:

(a) The filtrates of plasmas, regardless of their origins, were not only devoid of cytotoxicity but also somewhat stimulative of cellular metabolic function (Fig. 7). This probably implies the presence of some sort of enhancing factors in the ultra-filterable portion of plasma, as described earlier by Warren [285] and Sanford et al [227].

(b) Heating (at 56°C for 30 minutes) abolished cytotoxicity from

human plasmas. However, the apparently incomplete disappearance of cytotoxicity of some plasmas could not be readily explained. An attempt to restore cytotoxicity of heated human plasmas by adding either fresh autologous (rat) or xenogeneic (guinea pig) serum was not fruitful. Perhaps the two-stage incubation technique (11, 69, 250), in which cells are first incubated with heated plasma only and autologous, allogeneic or xenogeneic complement added later, could have yielded some restoration of cytotoxicity. In fact, in a few experiments done later for human lymphocytotoxicity study, the restoration of activity of heated antiserum was effected by this technique.

(c) The suppression of cytotoxicity by EDTA (Fig. 9) readily indicates the cation-dependency, particularly on Mg ion, of the systems involved, and constitutes a further evidence for immune cytolysis. The fall in the rate of C^{14}-RNA synthesis due to EDTA is not surprising inasmuch as the cells require divalent cations for their metabolic activities, and is in agreement with the earlier report of Lochte (160).

(d) The finding that the removal of properdin did not alter cytotoxicity of human plasmas (Fig. 10) indicates specificity of both natural xenogeneic and induced heterophile cytotoxicity systems.

(e) Experiments using plasmas made selectively devoid of C'3 or C'4 and their mixtures (Fig. 11) are conclusive of the complement dependency of the two cytotoxicity systems. Here again, however,

the fall in RNA-synthetic activity of cells incubated with autologous R3, R4 or R3 + R4 could not be given an adequate explanation.

Through the experiments cited above, so far no difference in the underlying mechanism was disclosed between the natural xenogeneic cytotoxicity and the induced heterophile cytotoxicity, both being an antibody-complement system.

However, as noted in Fig. 5, the relationship between heterophile cytotoxicity and allogeneic cytotoxicity in human serum seems to be of some interest. In the case of multiparas' sera, there was no positive association between the two types of cytotoxicity; that is, one could be demonstrated independently of the other. On the other hand, in the case of sera from the hemodialysed patients, there seemed to be a positive association between the two cytotoxic phenomena: that is, whenever one was present the other was always co-existent, although the number of sera tested was rather small.

Thus, whereas the data from the multiparas' sera are not of immediate clinical application except to suggest that these sera are of relatively narrow specificity, the positive association of two cytotoxic activities found in the sera of hemodialized patients may be of some practical value in that the demonstration of anti-rat bone marrow cytotoxicity might serve as an indicator for a concomitant presence of allogeneic lymphocytotoxicity.

These human antibodies reacting with rat cells are not Forssman antibodies since the rat is known to be a Forssman antigen free

species [34, 218].

7. Horse Anti-Human Lymphocyte Serum.

All of the horse anti-human lymphocyte serum preparations tested were the final products following several purification steps including absorption with human erythrocytes and salt fractionations. Table 6 reveals that in general the strength and frequency of reactivity of individual horse antisera paralleled each other. But a few horse sera showed some degree of specificity with respect to individual members of the cell panel. The most plausible explanation for this individual specificity may be that most of the species-specific and some of the individual-specific antibodies have been lost during the absorption with human erythrocytes which must have been contaminated by unknown numbers of lymphocytes. Therefore it is felt that each preparation of horse antihuman lymphocyte serum should be checked against lymphocytes of a prospective recipient as well as a panel of lymphocytes to ensure that both anti-individual and anti-species antibodies have not been removed inadvertently by the absorption steps. Correlation between clinical immunosuppressive effect and lymphocytotoxicity or any other *in vitro* tests has not been well established and awaits further investigations.

8. Lymphocytotoxic Antibodies in Pregnant Women.

In screening any given multipara's serum for the presence of

cytotoxic antibodies, the selection of size of cell panel consisting of 10 donors was arbitrary. However, the finding that most of antisera could be detected within the first several donors tested substantiates that the size of cell panel was adequate without missing many useful antisera. Ideally, each multipara's serum should be checked against lymphocytes from the corresponding husband. This was, however, found rather infeasible.

The technique of "mixed cell incubation" was devised by the candidate for further simplification of the screening procedure and was found to be very efficient and time-saving as illustrated in Table 7.

The induction of lymphocytotoxic antibodies by pregnancy is of great importance inasmuch as immune sera thus produced constitute the most abundant source of oligospecific antisera presently available for typing of human leukocytes. The incidence of such antibodies among pregnant women (Table 8) is also of interest and deserves a comment. With the screening procedure mentioned above, none of 20 primigravidas was shown to contain antilymphocyte antibodies. With the second and subsequent pregnancies the incidence of antibodies increased proportionally to the number of pregnancy, probably reflecting hyperimmunization effect.

Evidently the presence of lymphocytotoxic antibodies in the maternal circulation does not cause any deleterious effect on the

fetus. This impression is substantiated by the finding of Goodlin et al [81] that, in mice, maternal immunization to paternal antigens either by pregnancy or by spleen cell injection did not cause decreased fertility or fetal disease.

9. Lymphocytotoxic Antibodies Induced by Repeated Hemodialysis.

The role of hemodialysis in the incidence of antilymphocyte antibodies seems to be of a great significance in human renal transplantation, since practically all of chronic uremics are placed on intermittent hemodialysis while awaiting a renal allograft.

Table 9 shows the incidence of antilymphocyte antibodies in male and nulliparous female uremics. There seemed to be no positive correlation between the formation of such antibodies and the duration of intermittent hemodialysis in 34 patients studied. Possible factors predisposing to the development of antibodies under these circumstances are quite obscure. Although it is a possibility that everybody may develop antibodies if dialized for an indefinite period of time, some genetic factor could be responsible for determining between responders and non-responders as elaborated by Cinader et al [40] in mice.

On the other hand, a mention should be made of the finding by Dossetor et al [54] that, when immediate allograft failures were excluded, patients who were subjected to a longer period (over 6 months) of hemodialysis showed a lower rejection index during the

first three months after renal transplantation compared to those who underwent a shorter period (under 6 months) of hemodialysis. This difference may be due to a possible induction of enhancing antibodies by longer periods of hemodialysis.

10. Lymphocytotoxic Antibodies in post-transplant patients.

As shown in Table 10, antilymphocyte antibodies were not detectable in any of 33 renal allograft recipients whose grafted kidneys were functioning adequately under immunosuppressive medication.

Since all of these patients had been hemodialyzed prior to transplantation and 3 of them had been pregnant, the absence of antibodies from their sera cannot necessarily be interpreted to mean that antibodies had not been present before transplantation. In at least some of these subjects, antibodies which may have been reactive with lymphocytes from persons other than their respective kidney donors, might have disappeared from the circulation during the post-transplant period of immunosuppressive regimen.

The above supposition could be strengthened by a later observation on the activity of serial serum samples obtained from a kidney recipient, G.F., as shown in Figure 15. This patient's serum had been strongly reactive with lymphocytes from a large portion of random persons tested before he received a kidney from a cadaver donor whose lymphocytes were not reactive with his serum. However, the grafted kidney suffered bouts of major rejection necessitating its

removal 4 months following its transplantation, despite vigorous immunosuppressive measurements. He was then placed back on hemodialysis using buffy coat free bloods for the ensuing 5 months. It was interesting to note that sera obtained during this period of time were no longer found to contain antibodies to lymphocytes from a number of cell donors including those whose cells were reactive with his pre-transplant sera. An attempt was then made to challenge him using whole bloods to prime the dialysis coils, resulting in the reappearance of lymphocytotoxic antibodies in his serum. It is most likely in this patient that the pre-existing antibodies degraded and that further formation of antibodies was suppressed by immunosuppressive medication and was not revoked until the next antigenic stimulation.

11. Anti-cytotoxic Effect of Hydrocortisone and Heparin.

Among the factors which modify cytotoxic reaction, it seems important to discuss the action of hydrocortisone and heparin.

Hydrocortisone manifests anti-cytotoxic effect at very high concentrations (10 mg or more per ml of incubation mixture, Table 12), which are not comparable to clinical blood levels. However, it should be pointed out that in vivo effect of any pharmacological agent is dependent on the duration of its action as well as on its concentration, whereas with a short in vitro incubation higher concentrations of any test agent must be required for the manifestation of its action.

This also seems to be the case with heparin, though to a lesser extent.

The anti-cytotoxic action of heparin (Fig. 12 and Table 13) evokes one important consideration; that is, heparin should not be present in any in vitro cytotoxicity test in order to make the test as sensitive as possible. However, if only heparinized blood samples are available the two-stage incubation technique (11) will eliminate the heparin effect.

The anti-cytotoxic effect of hydrocortisone and heparin in vitro supports the finding by Kountz et al (136) that intrarenal injection of methyl-prednisone, heparin and actinomycin was more effective than oral or systemic administration in the treatment of renal allograft rejection in dogs.

12. Significance of Lymphocytotoxic Antibodies in Renal Allotransplantation.

The deleterious effect of preformed lymphocytotoxic antibodies on renal allografts was amply demonstrated in this work (see Table 14).

On the other hand, the presence of lymphocytotoxic antibodies in recipient's serum could serve to find a compatible donor, as illustrated in the case of I. A. in Table 14. Her serum has been found to react strongly with lymphocytes from 119 out of 120 individuals tested. The only negative reaction was with cells of one of her two brothers, whose kidney was therefore transplanted and has been functioning satisfactorily. The lymphocyte

antigen profiles of this patient and her donor brother were found to be identical by Dr. P.I. Terasaki, as well as by the candidate. However, there were others, among the positive reactors, whose lymphocyte antigen profiles showed no mismatch against Terasaki's 7 defined antigen groups and on all of unspecified antisera used as well (Table 16). This implies that there must be other antigens which are not detected by the presently available typing sera but wide-spread among humans and, which sensitized her so strongly that her serum reacts with practically every person's lymphocytes; her brother must lack these particular antigens.

The above observation would lead to the following conclusions: (i) In certain instances, the major lymphocyte group match is not a sufficient evidence for histocompatibility between two persons, (ii) For a recipient whose serum contains widely reacting antibodies, a rare negative crossmatch of his serum with donor's lymphocytes can be regarded as a significant evidence for compatibility.

13. Specification of Allogeneic Antilymphocyte Sera.

The method (Table 15) used in this work, without aid of a computer, to specify antisera with regard to antilymphocyte antibodies is purely tentative. Nevertheless, through a number of attempts to type lymphocytes from prospective donors and recipients of renal allograft, the panel of antisera thus specified proved to be reliable in most instances when the results were compared with Terasaki's reports. However, the as yet insufficient number of specified anti-

sera and the possible presence of uncharacterized antibodies in many of them created a considerable ambiguity in determining the lymphocyte antigen profile of some individuals.

On the other hand, those antisera which are not readily specified may be very important. It was often found that a pair of cell donors were well matched against the defined antigen groups, yet showed mismatches against some of the unspecified antisera. This was more frequently so between unrelated persons than within sibships (Tables 17 and 18). Perhaps the latter situation requires relatively few sera for typing since only four different phenotypes would be possible among siblings in the absence of recombination, as stated by Amos et al [8, 12], Cepellini et al [38] and van Rood et al [270]. Thus, in random populations a good match against only the defined major antigen groups may not be sufficient for optimal histocompatibility, whereas within a family, especially between siblings, a match against the defined antigen groups may indicate optimal compatibility.

Therefore, it is felt that until a comprehensive panel of monospecific typing sera is available, unspecified antisera also should be used for histocompatibility testing.

GENERAL DISCUSSION ON HUMORAL ANTIBODIES

This chapter is devoted to a general consideration of the action of humoral antibodies. Although the investigative work reported in this thesis concerns mainly antigraft activity of antibodies, it seems appropriate to include in this discussion another biological effect of specific antibodies, namely, "enhancement" or "facilitation" of graft survival. These two opposite functions of antibody will be first described individually, then their relationship will be sought.

A. Graft-destroying Antibodies.

Arguments in favor of the role of humoral antibodies in graft rejection are as follows:

First, humoral antibody formation, once thought to be inconstant, now appears to be an invariable feature of immune response to allografts in all of the mammalian species tested (6, 96, 131, 199, 247, 281) and to be a frequent consequence of blood transfusion or pregnancy in human (45, 204, 205, 266, 273). These antibodies have been shown, in vitro, to mediate cytolysis of a wide variety of cell types, both normal and neoplastic, and it is probable that no cell type is completely resistant to this form of damage (240).

Second, humoral antibodies have been shown to pass into

diffusion chambers and mediate destruction of homografts inside without participation of host cells (10, 77, 278), and also shown to be elaborated by "immune cells" within such chambers and to effect accelerated destruction of grafts outside on the body surface (139, 195).

Third, humoral antibodies have been reproducibly shown to passively immunize a host against allografts in animals (239, 277).

Fourth, it has been amply shown, in the report by Terasaki et al (254) and in the data presented by the candidate (Table 14), that preformed lymphocytotoxic antibodies cause immediate rejection of renal allografts in man.

The above observations seem to point to a major role for conventional antibodies in the destruction of allografts. However, a question still remains as to the exact mode of action of antibody against graft in vivo; that is, whether antibody reaches a graft by perfusion, whether it is delivered as "cytophilic antibody" bound to lymphoid cells, or whether it is synthesized locally by immunologically competent cells.

B. Graft-enhancing antibodies.

Immunologic enhan cement has been defined by Kaliss (125) as a successful establishment or prolonged survival of an allograft as a consequence of the graft's contact with specific antiserum in

the host. The demonstrated requirement for contact of the graft with specific antibody as the initiating factor in immunologic enhancement distinguishes this phenomenon from other types of graft prolongation, designated by such terms as "acquired tolerance", "immune paralysis" and others (125).

Although this phenomenon was first clearly demonstrated in experiments involving tumor transplantation (128, 129) and has since been studied extensively with similar systems, it was also observed with normal tissues (18, 31). However, enhancement has often been less easy to demonstrate with normal tissue grafts than with tumor grafts.

The enhancement phenomenon was shown to follow both active and passive immunizations of the host specifically against the grafts (125). For tumor, it was found to be more uniformly induced by passive than by active immunization (125). However, with skin allografts a higher degree of enhancement could be procured by active immunization whereas passive immunization was relatively ineffective (18, 31).

C. Factors determining between graft rejection and enhancement.

Since it has been shown that humoral antibodies can mediate either graft rejection or enhancement, an attempt will be made below to examine variables operative in conditioning a host toward either of these two quite opposite responses.

1. Factors related to graft.

(a) Relative vulnerability to cytotoxic antibody:

Immunologic enhancement is more easily demonstrated with some classes of tumors than others [84], the difference in degree bearing an inverse correlation with the extent to which a given tumor is sensitive to the cytotoxic action of antibody. Thus, enhancement has rarely been demonstrated for leukemia.

(b) Inoculation dose:

Regardless of the type of tumor grafted, the relative dose of tumor cells and antiserum may be a critical parameter in determining whether enhancement or graft destruction will ensue [27, 86]. For example, Boyse et al [27] defined the conditions for inhibition or enhancement for a leukemia, enhancement being produced when the ratio of tumor cell dose to antibody was sufficiently large. On the other hand, highly resistant tumors, such as sarcoma, can be made vulnerable to damage by an appropriately high ratio of antibody to tumor cell dose [183].

(c) Time of applying:

Either allograft rejection or enhancement could be demonstrated in actively immunized animals [67, 127]. If the initial stimulus was live allogeneic tumor graft (which was eventually rejected) a second graft of the same tumor inoculated within 5 to 14 days after the first inoculation exhibited an accelerated rejection. With increasing

intervals between the first and second graftings, a greater proportion of the hosts showed tumor enhancement. The temporal relation for these converse responses was considered to reflect a waning allograft resistance and a concomitant appearance and persistence of circulating (enhancing) antibody (125).

(d) Status of graft at the initiation of enhancement:

Kaliss (125) demonstrated a simultaneous occurrence of enhancement of a first tumor graft and second-set rejection of a second graft in the same mouse, whether it had been actively or passively immunized. Apparently the status of the graft at the initiation of enhancement was a contributing element. The most obvious features distinguishing the first graft from the second were its larger size, its longer residence and its established vascularization.

(e) Immunogenicity of tissue:

Tissues vary in their enhancing activity, certain tumors (particularly sarcoma I), spleen and parotid glands being the best sources (141).

2. Modes of immunization.

Kaliss (126) stated that the tactics for immunization to induce enhancement do not differ in essence from usual procedures for producing immunity in other situations. However, there have been many publications reporting different effects of varying immunization

procedures in conditioning the host. Therefore, some of these factors will be examined below.

(a) Viability of tissue:

When the initial immune stimulus was a live tumor graft, a second graft of the same tumor is either rejected in an accelerated fashion or enhanced, depending on the time interval between the first and second graftings as mentioned earlier [127]. On the other hand, when killed tissue (e.g. freeze-dried tumor or normal tissue) was the initial immunizing material, enhancement rather than graft rejection occurred with a subsequent tumor inoculum. Increased resistance to a subsequent graft, however, could be demonstrated if the dose of killed tissue and the time interval to grafting were carefully manipulated [123]. Judging from these observations, there seems to be no fundamental difference between live or killed tissues as to their effect in conditioning a host. In general, however, for reasons not as yet understood, killed tissue is less effective than a live graft in inducing a "second-set" response [125]. In other words, the killed tissue seems to induce a good humoral response, which is the essential prerequisite for enhancement.

(b) Antigen dose:

A dosage phenomenon was demonstrable for some graft-host combination , very small doses of freeze-dried tissue inducing heightened resistance in the host, while large doses permitted enhancement [124].

(c) Route of antigen administration and immunization schedule:

With respect to these points, the following observations made by Medawar et al [31, 169] are notable. First, the sensitizing effect of leukocytes was at least 18 times greater through intradermal route than through intravenous in rabbits [169]. But there was no mention about a possible enhancing effect of the intravenous route of immunization. Second, in mice, repeated intraperitoneal injection of 25 to 125 million allogeneic lymphoid cells diminished the sensitivity of their recipients more effectively than a single injection, and the antiserum thus produced caused some prolongation of skin allograft survival in passively immunized animals [30, 31].

Although several variables operative in conditioning a host toward either graft rejection or enhancement have been examined above, it should be stressed that there seems to be no one model system which would result in the same effect, either graft rejection or enhancement, in all graft-host combinations. In fact, Gorer [84] has stated that one can only construct a theory of enhancement provided that the model is a single tumor-host combination and variations do occur with different tumor-host relationships.

3. Qualitative differences in biological activities of antibodies present in a single antiserum.

Perhaps the most plausible explanation for the two fundament-

ally different effects of antiserum, i.e. graft rejection and enhancement, may reside in the qualitatively different biological activities exerted by different antibodies contained in a single antiserum, as suggested by Kaliss (126), who quoted the following observation made by Brent and Medawar (31). Antisera recovered from mice which have received allografts can cause hemagglutination, hemolysis or complement fixation and can exercise cytotoxic or protective action. It cannot be assumed that all these effects are simply different manifestations of the action of a single class of antibody; for example, there is some evidence for a distinction between agglutinins and lysins (32) and between enhancing and complement-fixing antibodies (130).

Recently, Voisin et al (275) made an attempt to ascertain, in mice, whether three immunological effects, i.e. hemagglutination, cytotoxicity and enhancement, are attributable to one immunoglobulin acting under different conditions, or if two or more immunoglobulins are involved. By electrophoresis of antiserum, they separated 3 immunological activities: cytotoxic activity in the slower fractions, enhancing activity in the faster fractions (almost without detectable overlapping) and hemagglutinating activity in almost the whole spectrum. However, their results did not allow firm conclusions as to the identification of the responsible immunoglobulin for each activity.

The theory that two opposing immunological activities, cyto-

toxicity and enhancement, may reside in separate immunoglobulin molecules of a single antiserum allows one to speculate that the relative quantity of these two kinds of molecules is crucial for the type of immune reaction, either rejection or enhancement, occurring in any given host-graft combinations [276].

SUMMARY AND CONCLUSIONS

In vitro cytotoxicity of human serum on xenogeneic and allogeneic cells was studied. Xenogeneic cells were from rat's bone marrow and spleen, and allogeneic cells used were human peripheral lymphocytes. The human subjects, from whom serum was obtained, could be classified into two groups: those without history of exposure to human tissue antigens and those with such exposure which might have caused immune response. The former consisted of healthy males and nulliparous females and non-transfused, non-dialysed uremic patients; the latter, multiparous women, multi-transfused patients, uremic patients with repeated hemodialysis, and renal allograft recipients.

In the study of xenogeneic cytotoxicity system, two techniques, trypan blue staining and C^{14}-uridine incorporation, were used to measure cell viability. These two parameters were found to correlate significantly with each other in general. However, there was a considerable amount of discrepancy between them in some instances, due probably to complete disruption of some of the injured cells which consequently were not observable as stained by trypan blue. In the study of allogeneic lymphocytotoxicity system, only trypan blue staining technique was used to determine cell death.

The xenogeneic cytotoxicity study revealed the following observations:

1) Human serum contains natural xenogeneic cytotoxicity on rat bone marrow and spleen cells, as indicated by toxic action of sera from healthy males and nulliparous females.

2) Sera from some of the human subjects who have been exposed to human tissue antigens in one way or another, exerted additional cytotoxicity beyond the mere natural xenogeneic toxicity, indicating the presence of heterophile antibodies.

3) Through the study of factors modifying cytotoxic reaction, it could be concluded that both natural xenogeneic and induced heterophile cytotoxicities were mediated by antibody-complement system.

The allogeneic cytotoxicity study provided the following information:

1) Sera from humans without history of exposure to human tissue antigens do not contain allogeneic lymphocytotoxic antibody; that is, there seems to be no natural allo-antibodies directed against lymphocytes in human serum.

2) A certain percentage of humans who have been exposed to human tissue antigen by means of pregnancy, blood transfusion or hemodialysis, possess allogeneic lymphocytotoxic antibodies. Particularly in the case of pregnancy, a direct correlation is evident between the incidence of antibodies and the parity.

3) The presence of preformed lymphocytotoxic antibodies,

through whatever means, in the circulation of allograft recipient is extremely detrimental to grafted organ from a donor whose lymphocytes react with the recipient's serum. However, there seem to be instances in which these antibodies could serve to reveal an optimal histocompatibility if a direct crossmatch between recipient's serum and donor's lymphocytes is negative.

4) Hydrocortisone and heparin exert anti-cytotoxic action in vitro with a good dose-response relation, indicating their possible use, at high dosages, in the management of allograft rejection.

5) Specification of allogeneic antilymphocyte sera with respect to defined lymphocyte antigen groups using a panel of pretyped cell donors, is plausible and reliable. This method would enable one to bypass the complicated statistical analysis of antisera in establishing a local panel of tissue typing sera.

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LIST OF TABLES

LIST OF TABLES (Cont'd)

COMPARISON OF VARIOUS METHODS FOR SEPARATION OF LYMPHOCYTES FROM WHOLE BLOOD.

Method / Efficiency \ No.of Experiment	Method i*	Method ii	Method iii	Method iv
	11	8	10	11
Percentage Yield of Lymphocytes	34.0 ± 4.2**	24.4 ± 2.7	25.6 ± 2.8	28.2 ± 3.4
Percentage Purity of Lymphocytes	79.3 ± 6.7	93.8 ± 4.4	93.6 ± 1.9	95.0 ± 1.0
Percentage Erythrocytes Contamination	55.3 ± 7.8	36.3 ± 3.1	43.8 ± 5.3	11.2 ± 1.6

* = See text for description of each method.
** = Mean ± standard error.

- Table 1 -

LYMPHOCYTE PREPARATIONS FROM WHOLE BLOOD OF THREE DIFFERENT GROUPS OF HUMANS.

(Prepared by Method iv.)

Group / Efficiency / No.of Experiment	Healthy	Hemodialysed	Cadaver Kidney Donor
	11	10	5
Percentage Yield of Lymphocytes	28.2 ± 3.4*	32.4 ± 2.6	28.0 ± 4.0
Percentage Purity of Lymphocytes	95.0 ± 1.0	80.6 ± 1.9	61.2 ± 5.7
Percentage Erythrocyte Contamination	11.2 ± 1.6	63.3 ± 7.4	53.5 ± 6.2

* = Mean ± Standard Error.

- Table 2 -

COMPARISON OF RADIOACTIVITY COUNTS OBTAINED BY THREE DIFFERENT METHODS OF SAMPLE PREPARATION (C^{14}-Uridine Incorporation Study)

a) Comparison between suspension technique and hyamine solubilization

Experiment No.	Suspension (a)	Hyamine (b)	Ratio (a:b)
1.	7,600*	9.260	0.82
2.	1,840	2,200	0.83
3.	850	1,000	0.83
4.	750	960	0.78
5.	500	570	0.88

b) Comparison between suspension technique and filter paper technique

Experiment No.	Suspension (a)	Filter Paper (b)	Ratio (a:b)
6.	13,770*	12,490	1.10
7.	12,840	13,680	0.93
8.	4,980	4,630	1.06
9.	2,010	1,910	1.04
10.	1,770	1,710	1.04
11.	1,500	1,620	0.92
12.	1,420	1,330	1.06
13.	620	580	1.07

* Radioactivity counts per minute.

- Table 3 -

EXTENT OF CYTOTOXICITY OF HUMAN PLASMAS OR SERA ON RAT BONE MARROW CELLS MEASURED BY URIDINE-2-^{14}C INCORPORATION AND TRYPAN BLUE DYE EXCLUSION

No.[a]	Plasma or Serum	Origin[b]	Dilution[c]	Uridine-2-^{14}C, V_1(%) [d]	Trypan blue V_2(%)[d]	Trypan blue V_3(%)[d]
1	Plasma	Uremic	1:3	74.0	81.0	54.2
2	"	Normal	"	41.7	67.3	27.3
3	"	Gravid	"	35.3	56.7	58.5
4	"	Normal	"	33.7	59.3	32.8
5	"	Transplant	"	30.3	67.2	26.9
6	"	Dialysed	"	23.5	67.3	15.0
7	"	Transfused	"	23.2	39.5	19.0
8	"	Dialysed	"	11.3	8.3	11.5
9	"	Gravid	"	6.8	34.7	22.1
10	"	Transplant	"	5.5	7.2	3.2
11	"	Dialysed	"	1.7	67.7	15.0
12	"	Transfused	"	1.5	55.0	20.0
13	"	Dialysed	"	1.5	55.0	4.4
14	"	Gravid	"	1.5	0	0
15	"	Dialysed	"	1.1	7.5	8.8
16	"	Transfused	"	1.0	27.7	13.7
17	Serum	Dialysed	"	7.6	0	0
18	"	Gravid	"	3.6	3.7	1.3
19	"	Normal	"	3.3	15.3	8.9
20	"	Gravid	"	2.8	19.0	10.6
21	"	Normal	"	2.6	25.8	6.5
22	"	Gravid	"	0.8	0	0
23	"	Gravid	"	0.7	6.6	2.7
24	"	Gravid	"	0.2	0	0
25[e]	"	Gravid	"	0.4	0	0
26[e]	"	Gravid	1:9	1.3	2.1	1.1
27[e]	"	"	1:27	8.5	25.3	15.2
28[e]	"	"	1:81	43.3	55.0	44.5

a. Experiment number.

b. Origin of plasma or serum. Normal = Healthy persons without history of blood transfusion or pregnancy. Uremic = Uremic patients without history of blood transfusion. Gravid = Women during the second or subsequent pregnancy. Transfused = Patients with recent history of multiple blood transfusions. Dialysed - Chronic uremic patients on long term hemodialysis. Transplant = Recipients of kidney allograft.

c. Ultimate dilusion of plasma or serum in incubation mixture.

d. See the text for description.

e. Serial dilution of the same serum.

- Table 4 -

EFFECT OF HEPARIN ON CYTOTOXICITY OF HUMAN SERUM ON RAT BONE MARROW CELLS.

(Measured by C^{14}-uridine incorporation).

Experiment Number	Serum alone	Serum with Heparin							
		0.06[a]	0.08	0.12	0.3	0.4	0.6	0.8	1.2
1	30.5[b]					82.5			
2	30.0					84.5			
3	20.2					68.0			
4	14.1	30.0	47.2						
5	4.4	8.4	30.2						
6	3.6	19.6	19.6			40.0		60.0	
7	2.7			15.3	38.5				
8	2.6	3.6			20.0		39.5		
9	2.0			14.5	50.0				
10	1.5	13.1	47.6						
11	0.2						6.8		89.0

a = Concentration of Heparin (mg/ml) in incubation medium

b = Percentage cell viability.

- Table 5 -

REACTIVITIES OF HORSE ANTIHUMAN LYMPHOCYTE SERA (ALS) ON A PANEL OF HUMAN LYMPHOCYTES.

(Determined by Trypan Blue Staining).

ALS Batch No. / Cell Donor	1	2	3	4	5	6	7
J.O.	+++*	++++	+++	+++	+++	+	+++
I.T.	+	+++	++++	0	+	0	+++
I.A.	+	++++	++	0	++	+	++
D.D.	++	++++	++	++	++	++	+++
P.M.	++	++++	++	0	++	±	++
M.G.	++	+++	++	++	++	±	++
W.S.	+	++++	++	+	+	±	+++
Y.L.	+	++++	+	0	+	0	++
J.W.	+	+++	++	0	+	+	+++
G.C.	+	+++	+++	+	++	+	+++

*Degree of Positivity.

- Table 6 -

MIXED CELL INCUBATION FOR SCREENING OF SERA

AS TO THE PRESENCE OF LYMPHOCYTOTOXIC ANTIBODIES

(Determined by Trypan Blue Staining)

a) Reaction Pattern of a Negative Serum with 5 individuals' cells and their mixture

Cell donor \ Serum donor	H.M.
E.P.	6*
C.H.	0
J.W.	6
D.W.	0
M.Z.	0
Mixed Cells	0

*percentage of stained cells

b) Reaction Patterns of two Positive Sera with 3 individuals' cells and their mixture

Cell donor \ Serum donor	P.K.	D.W.
E.P.	23	3
E.S.	81	100
I.T.	74	84
Mixed Cells	38	43

- Table 7 -

INCIDENCE OF LYMPHOCYTOTOXIC ANTIBODIES

AMONG PREGNANT WOMEN

No. of Pregnancy	No. of Persons Tested	No. of Positive and Percentage	
1st	20	0	(0%)
2nd	20	3	(15.0%)
3rd	70	14	(20.0%)
4th	33	7	(21.2%)
5th	19	3	(15.6%)
6th	9	4	(44.4%)
7 - 9th	10	6	(60.0%)
10 - 15th	9	7	(78.0%)

- Table 8 -

INCIDENCE OF LYMPHOCYTOTOXIC ANTIBODIES AMONG PRE-TRANSPLANT PATIENTS ON LONG-TERM HEMODIALYSIS PROGRAM

(3 to 13 months)

	Positive	Negative	Total
Male	7 (24.1%)	22 (75.9%)	29
Nulliparous Female	1 (20.0%)	4 (80.0%)	5
Parous Female	2 (40.0%)	3 (60.0%)	5
Total	10 (25.6%)	29 (74.4%)	39

- Table 9 -

PRESENCE OF LYMPHOCYTOTOXIC ANTIBODIES

IN RENAL ALLOGRAFT RECIPIENTS

(3 months to 3 years after transplant)

	Positive	Negative	Total
Male	0	24	24
Nulliparous Female	0	6	6
Parous Female	0	3	3
Total	0	33	33

Table 10

LOSS OF LYMPHOCYTOTOXICITY FROM ANTISERUM DUE TO AGING AND RESTORATION OF TOXICITY BY TWO-STAGE INCUBATION

(Determined by Trypan Blue Staining)

Antiserum / Cells	Fresh	Aged (8 months)	
	One-stage Incubation	One-stage Incubation	Two-stage Incubation
I.A.	90*	0	86
W.M.	73	4	52

* Percentage of stained cells

EFFECT OF HYDROCORTISONE ON LYMPHOCYTOTOXIC REACTION IN VITRO, AND TOXICITY OF PRESERVATIVES ON CELLS

(Determined by Trypan Blue Staining)

Agent (mg/ml) \ Incubation medium		Normal sera[a]	Antisera[b]
None	(0)	0	90 - 100[c]
Steroid alone	(5)	0	67 - 100
	(10)	0	6 - 91
	(20)	0	0 - 20
Steroid with Preservatives	(5)	0	
	(10)	0 - 22	
	(20)	5 - 100	

a and b - Three sera from each category tested on five donors' cells

c - Range of percentage of stained cells

- Table 12 -

EFFECT OF HEPARIN AND PROTAMINE ON CYTOTOXICITY OF HUMAN ANTISERA ON HUMAN LYMPHOCYTES

(Determined by Trypan Blue Staining)

Medium / Agent (mg/ml)		Normal Sera[a]	Antisera[b]
None	(0)	0	100
Heparin	(0.7)	0 - 4[c]	0 - 35
	(7.0)	0 - 1	0 - 2
Protamine	(0.7)	0 - 8	78 - 100
Heparin and Protamine	(0.7) (0.7)	0 - 3	40 - 100
Heparin and Protamine	(7.0) (0.7)	0 - 2	0 - 6

a and b - Two sera from each category tested on three donors' cells

c - Range of percentage of stained cells

- Table 13 -

EVOLUTION OF DIRECT CROSSMATCH TEST (LYMPHOCYTOTOXICITY) AS APPLIED TO RENAL HOMOTRANSPLANTATION AT ROYAL VICTORIA HOSPITAL.

Recipient	Sex	Number of Pregnancies	Crossmatch	Type of Rejection	Histology of Kidney	Frequency of Reactivity positive/total
Stage 1 (Prior to September 1967) : No crossmatch						
I.A. (1)*	F	5		Immediate	Fibrinoid necrosis	(see below)
D.D. (1)	F	1		Immediate	Fibrinoid necrosis	(see below)
Stage 2 (September 1967 - December 1967): Retrospective Crossmatch.						
C.Mc.	F	3	0	None		0/13
J.W.	F	2	0	Major		0/10
G.R. (2)	F	0	+	Immediate	Fibrinoid necrosis	2/12
G.W.	M		0	None		0/12
J.C.	M		++	Immediate	Fibrinoid necrosis	10/10
Y.L.	M		0	Minor		0/10
D.D. (2)	F	1	+++	Immediate	Fibrinoid necrosis	(see below)
R.B.	M		0	Minor	Interstitial fibrosis	4/10
Stage 3 (December 1967 - February 1968): Prospective Crossmatch.						
I.A. (2)	F	5	0	None		119/120
D.D. (3)	F	1	0	Minor		16/23
G.F.	M		0	Major		10/13

* = Transplant number.

- Table 14 -

REACTIVITY OF 51 ANTISERA WITH RESPECT TO 7 MAJOR LYMPHOCYTE ANTIGEN GROUPS.

Antiserum	Lymphocyte Antigen Group (positive/negative)							Antibody	
	1	2	3	4	5	6	7	Probable **	Questionable*
RVH 23	1/1	1/7	1/11	1/1	1/7	1/8	0/1		
26	2/2	3/10	4/8	0/1	0/7	3/10	1/2		
29	1/0	2/2	2/4	0/0	0/2	1/3	0/1		
30	1/1	2/9	3/9	0/1	0/6	2/8	0/1		
31	1/3	2/11	3/14	0/1	0/8	2/13	0/2		
39	0/3	4/3	11/0**	0/0	2/2	10/4	2/2	3	
48	0/2	7/3	4/13	1/1	3/4	5/6	0/1		
49	0/3	2/10	2/16	0/1	0/7	2/13	0/1		
53	0/2	5/2	5/4	0/1	2/1	1/6	0/2		
58	0/2	1/4	4/7	0/0	0/3	2/4	0/1		
61	0/3	10/0**	6/9	0/1	4/3	5/8	0/2	2	
64	1/2	1/8	3/12	0/1	0/5	2/9	1/0		
74	0/1	3/3	1/11	0/0	1/3	2/7	0/1		
79	0/6	7/7	17/3*	2/1	0/7	9/7	1/3		3
84	0/1	2/4	1/9	0/1	1/2	1/4	0/1		
85	0/2	3/3	0/9	0/1	1/2	1/7	0/1		
87	4/1	11/2*	17/2*	2/1	6/3	18/0**	3/1	6	2,3
95	0/2	7/2	2/12	0/2	3/3	3/7	0/2		
96	0/5	13/1**	8/9	1/1	4/5	6/9	1/3	2	
103	0/2	0/8	0/11	0/1	4/5	0/9	0/1		
107	0/2	0/6	0/10	0/1	0/6	0/8	0/1		
110	0/0	0/1	0/8	1/1	0/1	0/5	0/0		
119	4/0	9/2*	7/7	2/1	8/1*	9/5	4/1		2, 5

- Table 15 -

Antiserum	Lymphocyte Antigen Group (positive/negative)							Antibody	
	1	2	3	4	5	6	7	Probable**	Questionable*
RVH 121	4/0	7/1*	7/6	2/1	8/1*	8/5	3/0		2,5
124	5/0**	7/0**	4/6	2/0	6/1*	8/4	5/0**	1,2,7	5
126	0/2	0/7	0/11	0/1	0/5	0/8	0/1		
127	0/2	0/7	2/14	0/1	0/4	1/8	0/1		
128	0/2	0/7	0/10	0/1	0/5	2/10	0/0		
129	2/2	10/1**	12/1**	2/1	4/1	9/2*	1/2	2,3	6
130	2/1	2/3	7/4	0/2	0/4	1/3	1/1		
135*	8/0**	3/2	0/6	0/1	0/2	1/5	1/0	1	
139	0/2	2/3	0/7	0/1	1/2	0/4	0/1		
140	1/1	1/3	1/8	0/1	0/3	0/5	0/0		
142	1/1	3/1	3/5	0/1	1/1	0/5	1/0		
143	1/2	0/4	7/4	0/1	1/3	10/2*	0/2		6
144	0/1	4/3	10/4	2/0	1/3	5/3	1/0		
145	1/3	8/0*	12/1**	2/1	5/2	11/2*	1/3	3	2,6
146	2/2	9/0*	12/0**	2/0	1/2	12/2*	1/2	3	2,6
156	8/0**	2/7	2/13	0/2	2/4	4/9	1/3	1	
161	1/3	7/0**	5/5	1/1	4/2	5/6	0/3	2	
163	0/2	0/7	0/10	0/1	0/5	0/7	0/1		
Duke B.M.	4/3	3/5	6/11	2/2	9/2*	7/11	4/1		5
J.T.	12/1	7/9	17/4*	3/0	4/7	13/4*	3/0	1	3,6
D.K.	3/2	10/0**	14/1**	2/0	7/1*	13/2*	3/2	2,3	5,6
B.C.	2/3	5/8	4/14	0/3	4/3	7/8	2/2		
R.A.	2/0	12/3*	8/7	1/0	7/1*	8/4	2/0		2,5
T.H.	8/0**	15/0**	12/8	3/0	7/2	12/6	8/1*	1,2	7
S.A.	3/1	7/7	12/6	1/2	3/6	18/2**	1/4	6	

- Table 15 (cont'd) -

Antiserum	Lymphocyte Antigen Group (positive/negative)							Antibody	
	1	2	3	4	5	6		Probable**	Questionable*
W.B	2/5	14/0**	7/12	2/1	5/4	7/11	1/4	2	
D.A.	6/1*	11/3*	19/2**	9/0**	6/2	17/3*	4/1	3,4	1,2,6
M.R.	0/3	3/7	3/12	1/0	1/6	3/10	0/2		

** = Positive with over 90% of a panel of 10 or more cell donors who contain the particular antigen, or positive with 100% of a panel of 5 to 9 cell donors who contain the particular antigen.

* = Positive with 75 to 90% of a panel of 10 or more cell donors who contain the particular antigen, or positive with over 80% of a panel of 5 to 9 cell donors who contain the particular antigen.

- Table 15 (cont'd) -

DISCREPANCY BETWEEN LYMPHOCYTE GROUP MATCH AND CROSSMATCH.

Typing Sera			Prospective	Prospective Donors		
	Specificities		Recipient			
Serum No.	Probable	Questionable	I.A.	W.M.	O.M.	G.C.
RVH 13			0	0	0	0
26			++	0	0	0
30			++	0	0	0
31			++	0	0	0
39	3		+++	+++	++++	+++
48			+++	++	0	0
53			0	0	0	0
61	2		+++	+	0	+++
74			++	0	0	±
79		3	+++	+++	+++	+
84			+	0	0	±
85			±	±	0	±
87	6	2,3	+++	+++	++++	++++
95			+	0	0	0
96	2		++	++	0	++
Duke B.M.		5	+++	+++	0	0
J.T.	1	3,6	++++	+++	+	0
D.K.	2,3	5,6	++++	+++	+++	++++
B.C.			0	0	0	0
R.A.		2,5	++	0	0	0
T.H.	1,2	7	++++	++++	0	+++
S.A.	6		++++	++	++++	0
W.B.	2		++++	+++	0	++++
D.A.	3,4	1,2,6	++++	++	++	++++
M.R.			+	±	0	0
Lymphocyte Group Match				Compatible	Compatible	Compatible
Crossmatch				Negative	Positive	Positive

- Table 16 -

EXAMPLE OF MATCHING BETWEEN SIBLINGS.

Against Specified Sera					Against Unspecified Sera.		
	Specificities		Siblings tested			Siblings tested	
Serum No.	Probable	Questionable.	I.M.	R.M.	Serum No.	I.M.	R.M.
RVH 39	3		++++	++++	RVH 26	0	0
61	2		0	0	48	0	0
79		3	++++	+++	49	0	0
87	6	2,3	++++	++++	53	0	0
96	2		0	0	58	0	0
119		2,5	0	0	64	0	0
121		5	0	0	74	0	0
124	1,2,7	5	0	0	84	0	0
129	2,3	6	++++	++++	85	0	0
135	1		0	0	95	0	0
143		6	+++	+++	103	0	0
145	3	2,6	++++	++++	107	0	0
146	3	2,6	++++	++++	110	0	0
156	1		0	0	126	0	0
161	2		0	0	127	0	0
Duke B.M.		5	0	0	128	0	0
J.T.	1	3,6	±	±	130	++	++
D.K.	2,3	5,6	+++	+	139	0	0
R.A.		2,5	0	0	140	0	0
T.H.	1,2	7	0	0	142	0	0
S.A.	6		++++	++++	144	±	±
W.B.	2		0	0	Duke B.C.	0	0
D.A.	3,4	1,2,7	+++	+++	M.R.	0	0
			Identical			Identical	

- Table 17 -

EXAMPLE OF MATCHING BETWEEN UNRELATED PERSONS.

Against Specified Sera					Against Unspecified Sera		
	Specificities		Persons tested			Persons tested	
Serum No.	Probable	Questionable	C.D.	J.M.	Serum No.	C.D.	J.M.
RVH 39	3		0	0	RVH 26	0	0
61	2		++	++	53	+	±
79		3	+++	++	58	0	0
87	6	2,3	+++	+++	64	±	±
96	2		+++	++	74	+	0
119		2,5	++++	++++	84	±	±
121		5	++	++++	95	+	±
124	1,2	5,7	+	+	130	+++	0
129	2,3	6	++	+	139	+	0
135	1		0	0	140	++	0
143		6	±	0	Duke B.C.	0	0
145	3	2,6	++++	++++			
146	3	2,6	++++	+++			
156	1		±	0			
161	2		++	+			
Duke B.M.		5	0	0			
J.T.	1	3,6	0	±			
D.K.	2,3	5,6	++++	++++			
R.A.		2,5	+++	+++			
T.H.	1,2	7	++++	+++			
S.A.	6		0	0			
W.B.	2		++++	++++			
D.A.	3,4	2,1,6	0	0			
			Identical			Not identical	

- Table 18 -

LIST OF FIGURES

LIST OF FIGURES (Cont'd)

Figures

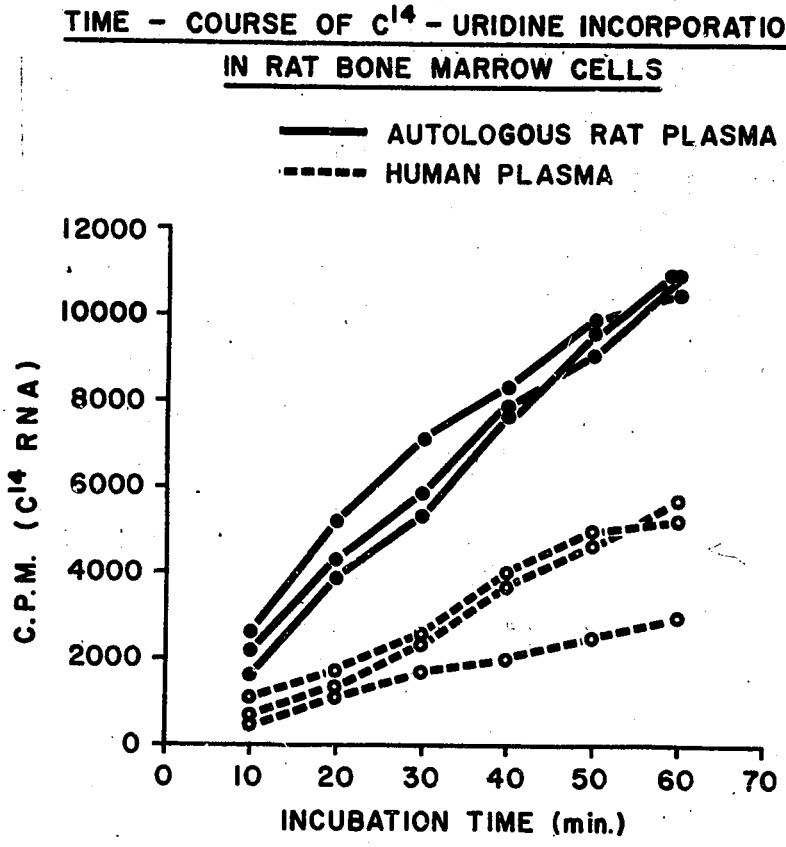
TIME - COURSE OF C14 - URIDINE INCORPORATION
IN RAT BONE MARROW CELLS
AUTOLOGOUS RAT PLASMA
HUMAN PLASMA
C.P.M. (C14 RNA)
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40
50
60
70
INCUBATION TIME (min.)

Figure 1

CORRELATION BETWEEN C^{14}-URIDINE INCORPORATION (V_1) AND DYE EXCLUSION (V_2 or V_3)

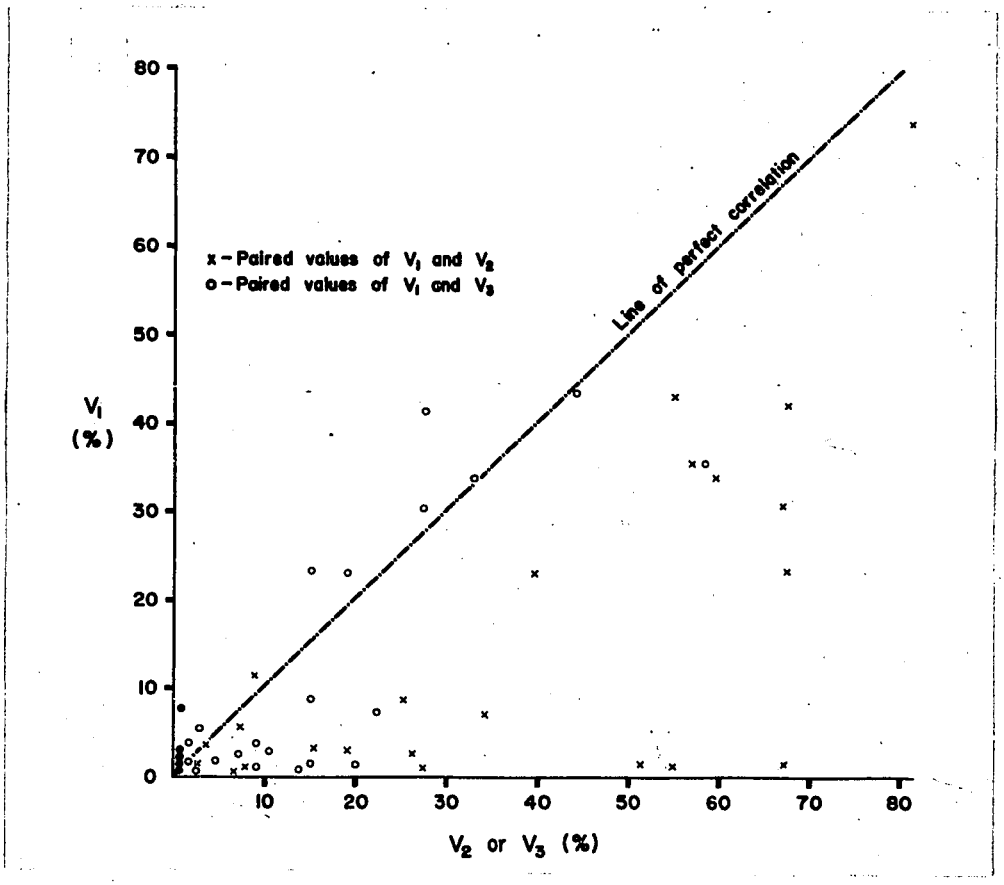

Figure 2

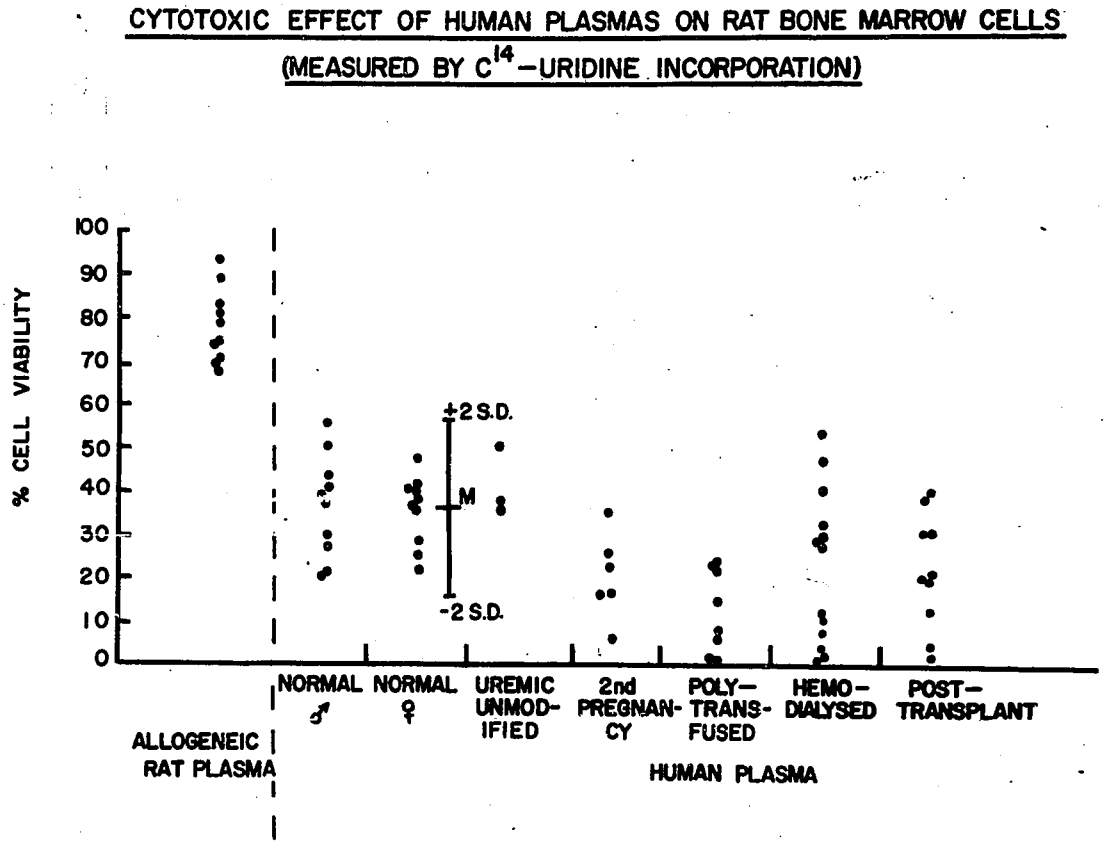
CYTOTOXIC EFFECT OF HUMAN PLASMAS ON RAT BONE MARROW CELLS
(MEASURED BY C14–URIDINE INCORPORATION)
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0
+2 S.D.
M
-2 S.D.
NORMAL ♂
NORMAL ♀
UREMIC UNMODIFIED
2nd PREGNANCY
POLY–TRANSFUSED
HEMO–DIALYSED
POST–TRANSPLANT
ALLOGENEIC RAT PLASMA
HUMAN PLASMA

Figure 3

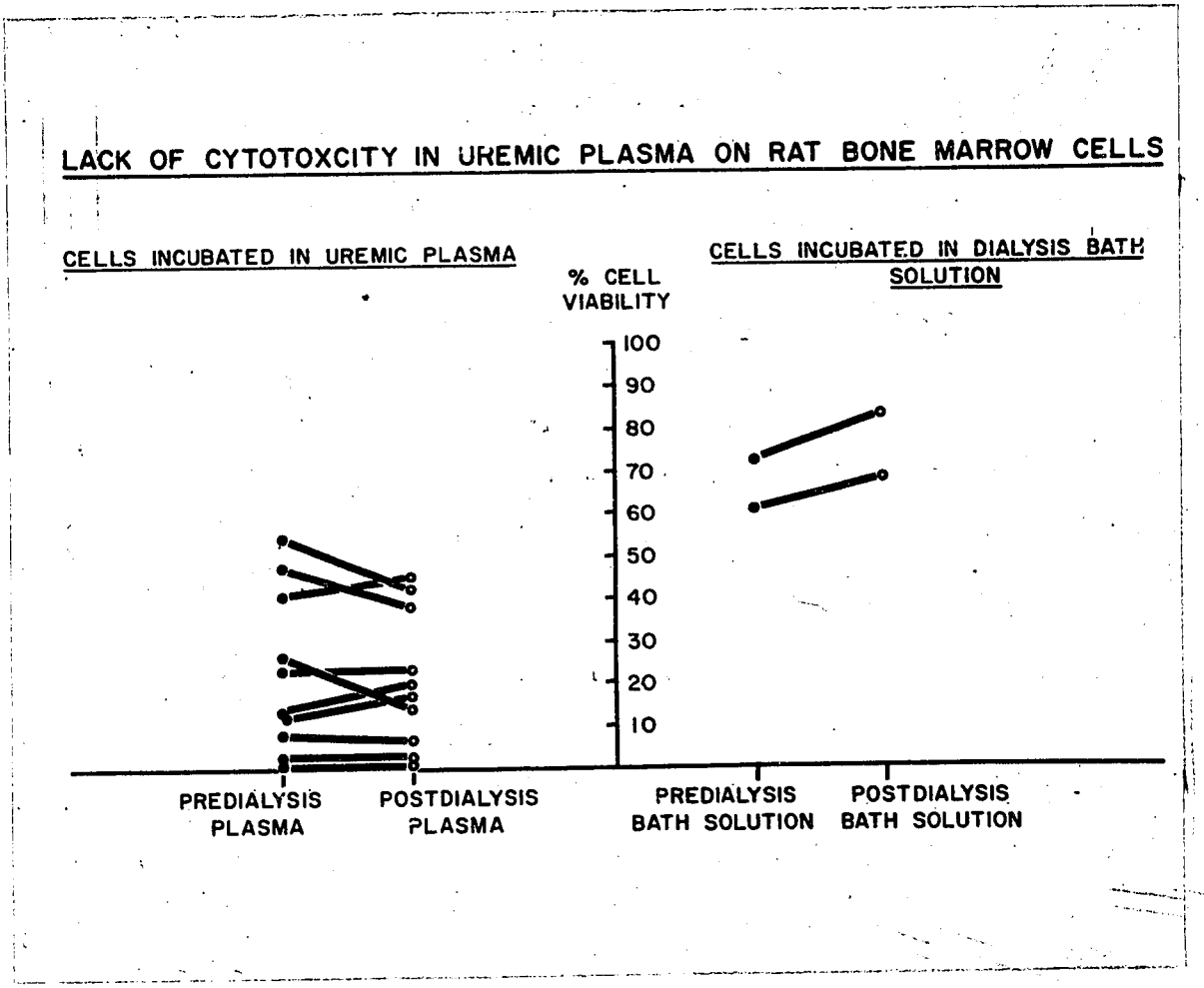
LACK OF CYTOTOXCITY IN UREMIC PLASMA ON RAT BONE MARROW CELLS
CELLS INCUBATED IN UREMIC PLASMA
% CELL VIABILITY
CELLS INCUBATED IN DIALYSIS BATH SOLUTION
100
90
80
70
60
50
40
30
20
10
PREDIALYSIS PLASMA
POSTDIALYSIS PLASMA
PREDIALYSIS BATH SOLUTION
POSTDIALYSIS BATH SOLUTION

Figure 4

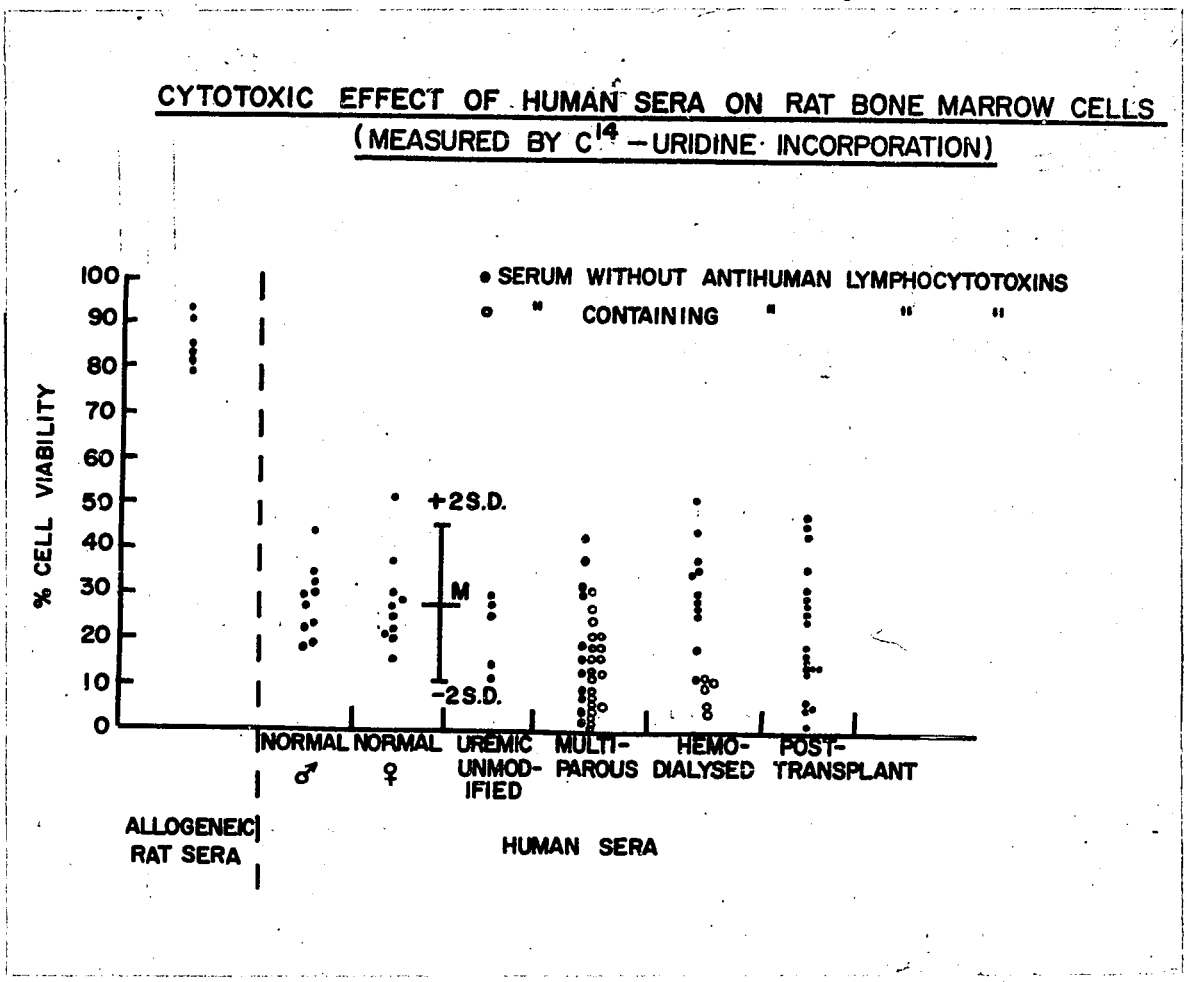
CYTOTOXIC EFFECT OF HUMAN SERA ON RAT BONE MARROW CELLS
(MEASURED BY C14 – URIDINE INCORPORATION)
• SERUM WITHOUT ANTIHUMAN LYMPHOCYTOTOXINS
o " CONTAINING " " "
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0
+2S.D.
M
-2S.D.
NORMAL ♂
NORMAL ♀
UREMIC UNMODIFIED
MULTIPAROUS
HEMODIALYSED
POST-TRANSPLANT
ALLOGENEIC RAT SERA
HUMAN SERA

Figure 5

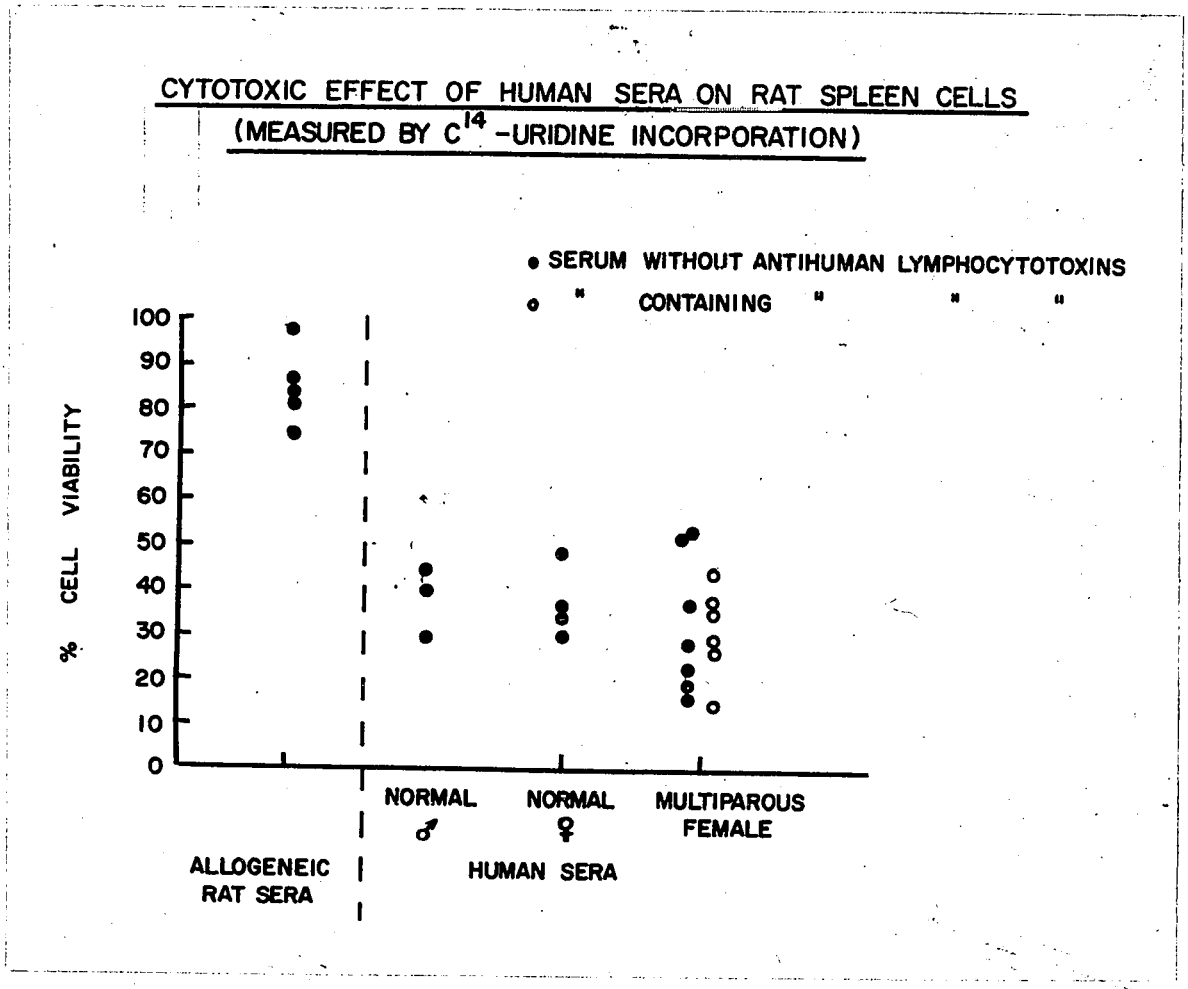
CYTOTOXIC EFFECT OF HUMAN SERA ON RAT SPLEEN CELLS
(MEASURED BY C14 -URIDINE INCORPORATION)
● SERUM WITHOUT ANTIHUMAN LYMPHOCYTOTOXINS
○ " CONTAINING " " "
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0
NORMAL ♂
NORMAL ♀
MULTIPAROUS FEMALE
ALLOGENEIC RAT SERA
HUMAN SERA

Figure 6

COMPARATIVE CYTOTOXICITY OF HUMAN PLASMA AND ITS FILTRATE ON RAT BONE MARROW CELLS

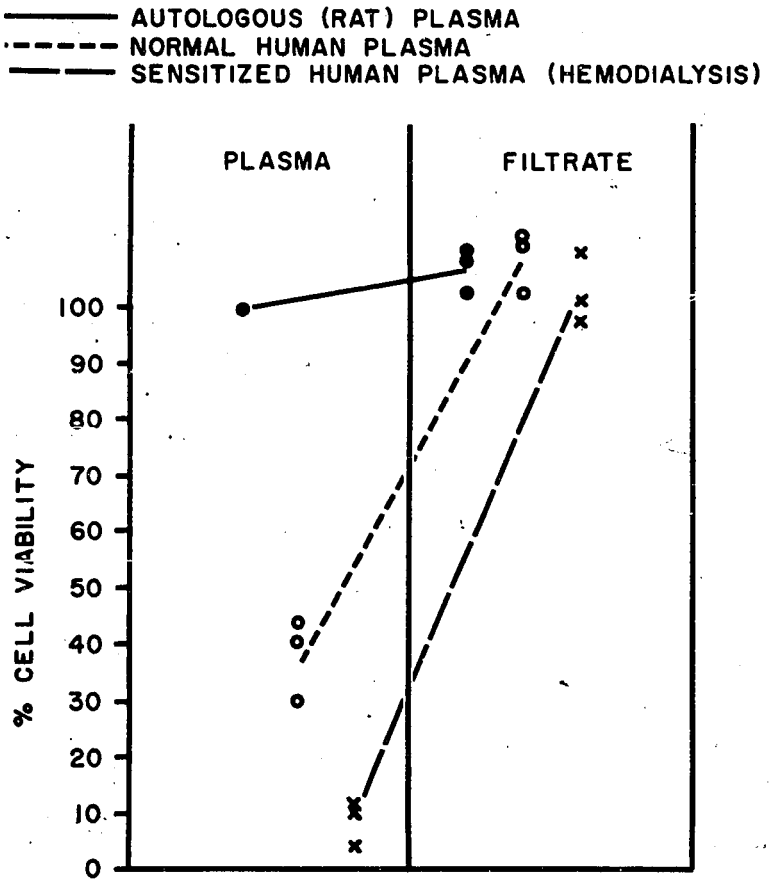

Figure 7

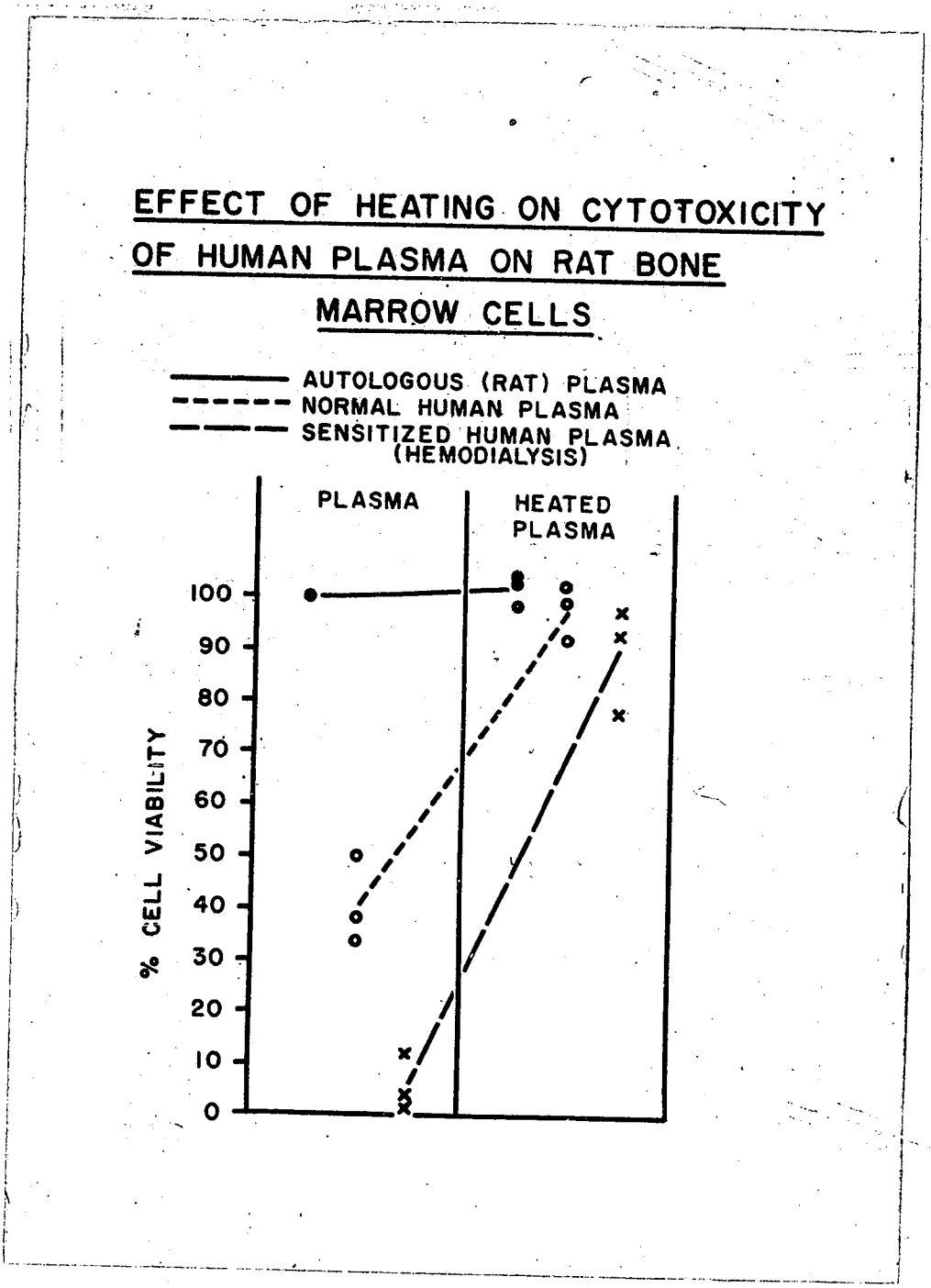
EFFECT OF HEATING ON CYTOTOXICITY OF HUMAN PLASMA ON RAT BONE MARROW CELLS
AUTOLOGOUS (RAT) PLASMA
NORMAL HUMAN PLASMA
SENSITIZED HUMAN PLASMA (HEMODIALYSIS)
PLASMA
HEATED PLASMA
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0

Figure 8

EFFECT OF EDTA ON CYTOTOXICITY OF HUMAN PLASMA ON RAT BONE MARROW CELLS

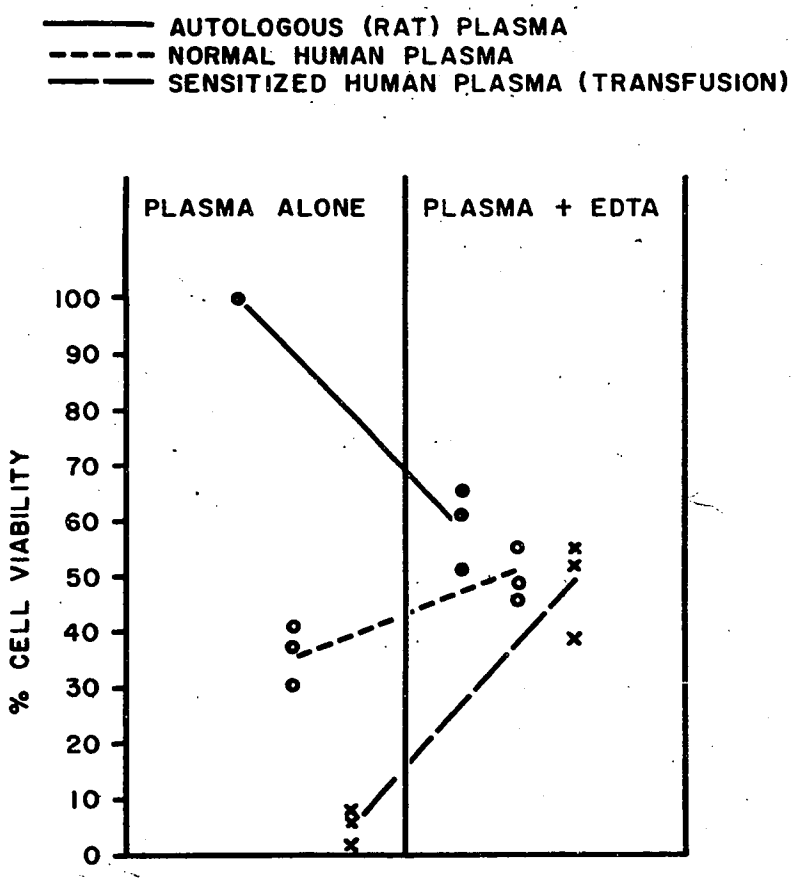

Figure 9

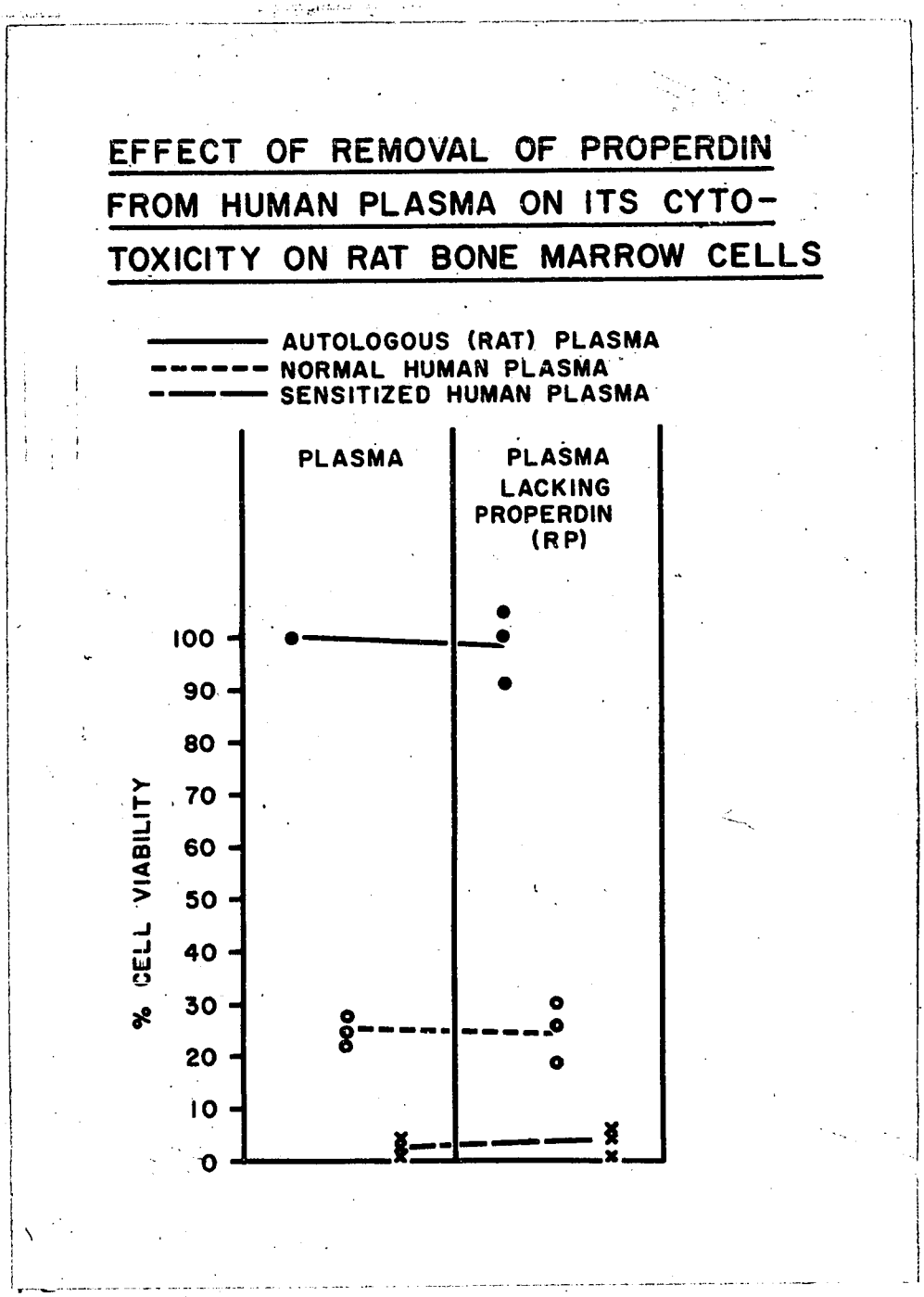
EFFECT OF REMOVAL OF PROPERDIN FROM HUMAN PLASMA ON ITS CYTO-TOXICITY ON RAT BONE MARROW CELLS
AUTOLOGOUS (RAT) PLASMA
NORMAL HUMAN PLASMA
SENSITIZED HUMAN PLASMA
PLASMA
PLASMA LACKING PROPERDIN (RP)
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0

Figure 10

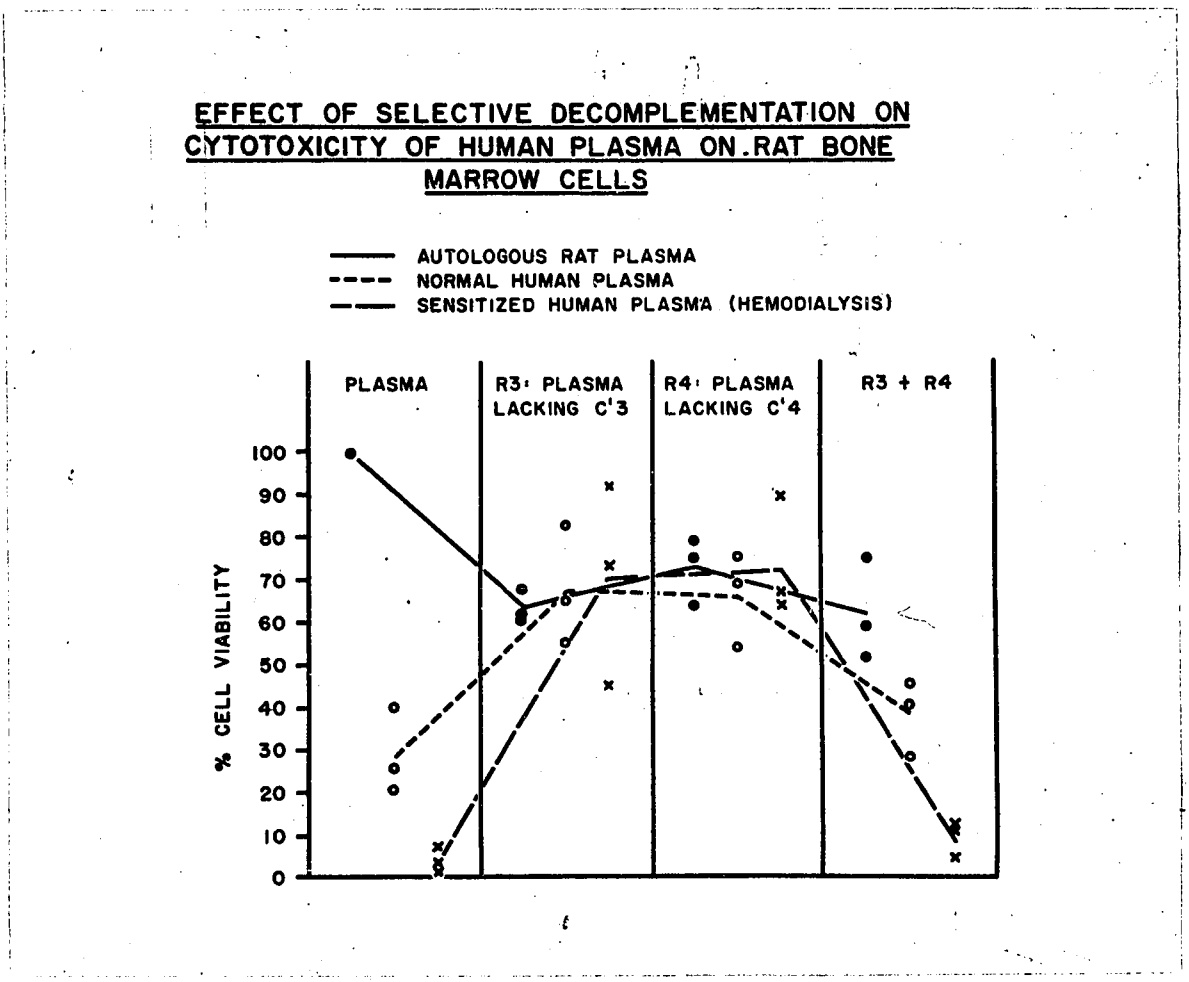
EFFECT OF SELECTIVE DECOMPLEMENTATION ON CYTOTOXICITY OF HUMAN PLASMA ON RAT BONE MARROW CELLS
AUTOLOGOUS RAT PLASMA
NORMAL HUMAN PLASMA
SENSITIZED HUMAN PLASMA (HEMODIALYSIS)
PLASMA
R3+ PLASMA LACKING C'3
R4+ PLASMA LACKING C'4
R3 + R4
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0

Figure 11

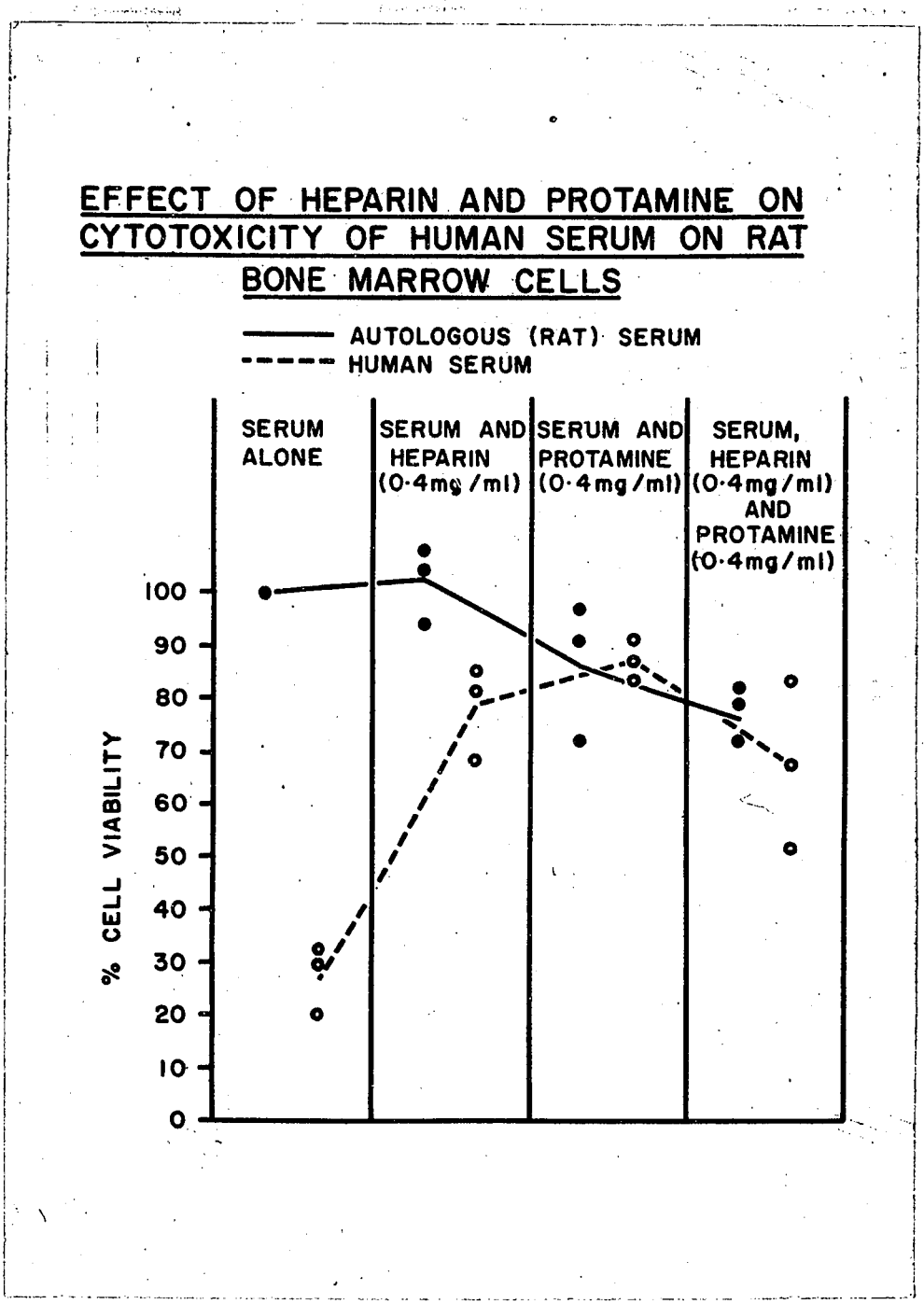
EFFECT OF HEPARIN AND PROTAMINE ON CYTOTOXICITY OF HUMAN SERUM ON RAT BONE MARROW CELLS
AUTOLOGOUS (RAT) SERUM
HUMAN SERUM
SERUM ALONE
SERUM AND HEPARIN (0·4mg/ml)
SERUM AND PROTAMINE (0·4mg/ml)
SERUM, HEPARIN (0·4mg/ml) AND PROTAMINE (0·4mg/ml)
% CELL VIABILITY
100
90
80
70
60
50
40
30
20
10
0

Figure 12

Renal tissue from an allograft removed from a patient (I.A.) 24 hours after implantation, showing fibrinoid necrosis of interlobular artery and glomurular pole. (Stained by phosphotungustic acid-hematoxylin).

Figure 13

Glomerulus from the same kidney (Fig.13), showing fibrinoid necrosis of afferent arteriole extending into glomerular tufts. (Stained by phosphotungustic acid-hematoxylin).

Figure 14

FLUCTUATIONS IN LYMPHOCYTOTOXIC ACTIVITY
IN SERUM OF A RENAL ALLOGRAFT RECIPIENT

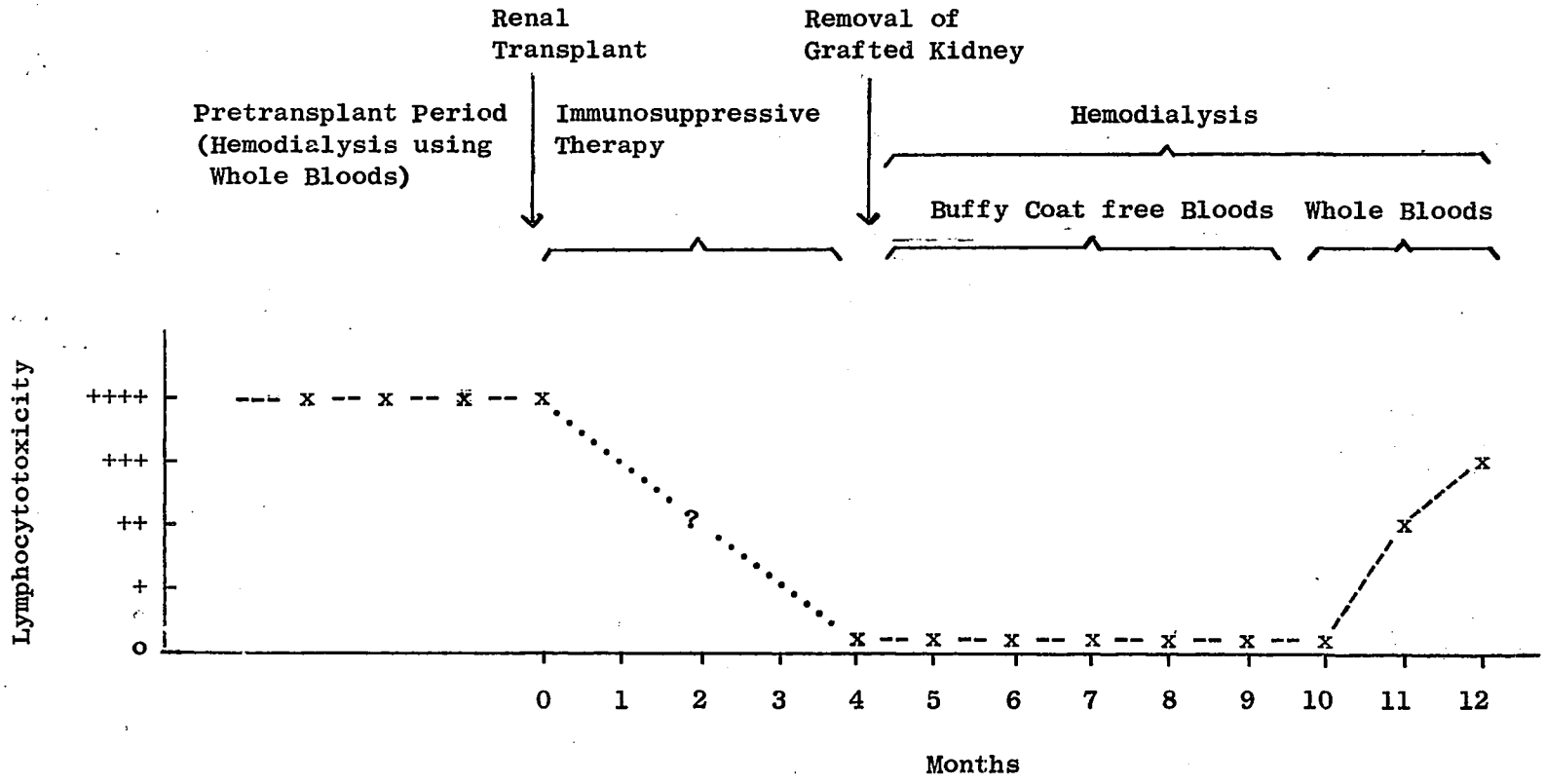

Figure 15