ALLOGRAFT ENHANCEMENT IN A RAT HEART TRANSPLANT MODEL

EFFECT OF T AND B CELL IMMUNIZATION

BY

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B.Sc., University of British Columbia 1972
M.D., University of British Columbia 1975

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENT OF THE DEGREE OF

MASTER OF SCIENCE

IN

THE DEPARTMENT OF SURGERY

We accept this thesis as conforming to the required standard

The University of British Columbia

JULY 1985

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ABSTRACT

Acute and chronic rejection continue to be the most important problems in maintaining a functioning organ allograft. Despite advances in immunosuppression, organs are still damaged or destroyed by the recipient's immune system. In order to protect the transplanted organ it is necessary to overwhelm the host's immune system and thus expose the host to the complications of invasion from fungi, bacteria, protazoa, reduction in oncologic surveillance, and reduction of stem cell production.

Donor specific immunosuppression would provide graft protection and allow maintenance of the host's immunologic competence.

Graft enhancement has been described for many years. Current practice uses this principle by pretransfusing prospective kidney transplant recipients with type specific blood. Previous work has supported the concept that this clinical effort can be produced by certain cells in the blood, specifically, lymphocytes.

To study the effects of preimmunization with T or B lymphocytes and platelets in a rat heterotopic heart transplant model, the following experiments were performed.

Experiment 1: The effect of pretransplant immunization with $1x10^7$ donor specific T cells or B cells showed that T cells have little affect on rejection of heterotopic heart allograft and B cells caused

prolongation of graft function. This effect is species specific and not due to a pure anti-idiotype phenomenon.

Experiment 2: The effect of heating the purified T and B cells at 56°C for 30 minutes is known to denature the presenting protein antigens on the cell membranes without destroying the cell membrane.

After pretransplant immunization, seven days prior to heterotopic heart transplant in the rat model, the previously observed prolongation of graft survival after nonheated B cell immunization was still present but not as marked.

Experiment 4: The effect of pretransplant immunization of donor specific T and B cells treated by heating to 85°C for 10 minutes followed by heterotopic heart graft showed that there was a significant prolongation of the engrafted heart following immunization with the denatured B cells. Cellular proteins are denatured by this pretreatment but polysaccharides are not.

Experiment 5: The effect of pretransplant immunization with purified donor specific platelets followed by heterotopic heart rat transplant showed no prolongation or shortening of graft survival.

It is concluded that, in the heterotopic rat heart transplant model, immunization with purified T cells 7 days prior to transplant has little effect on rejection. When B cells are immunized in the same way, graft survival is prolonged. If the cells are heated to 56°C for 30 minutes this effect is reduced but not eliminated. This effect indicates that

denaturation of protein HLA antigens on the presenting cell surface reduces the enhancing effect of the intact antigens on B cells. By denaturing, the presenting B cell protein graft enhancement is still present, suggesting the phenomenon of graft enhancement is not totally dependent on protein antigens but may have a contribution from mucopolysaccharides or other carbohydrates.

Donor specific purified platelet pretransplant immunizations produced no statistically significant prolongation of either PVG or F_1 heart grafts. This observation is consistent with the findings that purified T cell immunization do not produce graft enhancement.

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DEDICATION

To Trevor, March 3, 1985

ACKNOWLEDGEMENTS

I would like to thank those members of the Department of Surgery, University of Cambridge, for their aid in my research.

First, Professor Roy York Calne who was a constant source of inspiration and an ideal role model.

Second, Professor Allan Davies who's guidance in the field of immunology was of great importance.

Third, Gabsi Brons who performed the cell staining and demonstrated the separation techniques.

Fourth, Michael Butts, East Anglian Regional Health Authority, who performed the statistical analysis.

Finally, Doctor Derrick Wight who performed the pathological preparations.

INTRODUCTION

ALLOGRAFT PROLONGATION

Prolongation of allograft survival is the fundamental problem in clinical transplantation.[42,69] There are three methods of prolonging allograft survival: host immunosuppression, inducted host tolerance, induction of graft enhancement. Suppression of the the host's immune system by chemical or physical means is the current practice. This would include agents such as azathioprine, corticosteriods or cyclosporine. The problem with these agents is they are nonspecific and have broad actions thus making the host vulnerable to infections and neoplastic development as well as a great number of other undesirable side effects.[7]

Tolerance was first recognized in fraternal twin cattle who shared their fetal circulation.[4] Thus, the immature immune systems shared cells common to both fetuses. In adulthood these animals would not reject skin graft transfers. These were named chimeras.[16] The existence of tolerance is vital so that the host's immune system is tolerant to itself. Failure of phenotypic self tolerance is one theory for the mechanism of autoimmune disorders.[22] Studies have demonstrated that tolerance is an active process which involves direct contact between self components and specific antigen-reactive cells.[21,46] The antigen has generally been thought to be a protein.[13,14,83]

There are two types of tolerance: central and peripheral. In the central forms there is no antibody produced after antigenic challenge, since

the responsible immunocytes are missing (clonal deleation) or are silenced directly (clonal anergy).[6] In peripheral forms some antibody is produced but because of the expression of the suppression mechanism, antibody production is decreased or masked so that it is ineffective. For example, the production of certain antibodies simultaneously induces T suppressor cells to suppress the immunocompetent cells, the induction of anti-idiotypes or the production of immune compleses which do not have the ability to bind to effector cells.[25]

The third method of graft prolongation is graft enhancement.[47,49,79,87] After immunization with foreign tissues, antibodies may be formed that prevent graft rejection. Enhancing antibodies may coat the target graft or combine with released antigen to form immune complexes that react with lymphoid cells and prevent the immune response. Suppressor (T lymphocytes) cells may be generated by alloantigen stimulation,[80] thus suppressing the host immune system to react with the specific antigen. It has been shown that this is a very specific process.[81] A response against the full array of antigens is not necessary.[16] In renal allograft recipients, an important clinical observation has been made: grafts survive longer in patients who received multiple blood transfusions without becoming strongly sensitized.[101]

Thus the clinical promise of naturally induced graft tolerance has been demonstrated.

There are three groups of lymphocytes in the peripheral circulation: B lymphocytes (bursa derived), T lymphocytes (thymic derived) and N lymphocytes (null cells or non B or T by antibody staining technique).

The T lymphocytes are those lymphocytes thought to be responsible for cell mediated immunity (delayed hypersensitivity).[71] T lymphocytes respond to chemical messages (i.e. interlukens) and processed antigens. They may help the cell mediated response by inducing T cell conversion (T helper cell) to suppress this response (T suppressor cells) or act directly by destroying antibody coated cells.[2]

The B cells produce the antibody in response to an antigen (either direct or induction) but usually after the antigen has been processed (antigen presenter cell) by a T lymphocyte.[19]

Lymph nodes and spleen contain large numbers of both B and T lymphocytes.[33]

Fundamental questions to be addressed here are:

- Can enhancement be achieved without sensitizing the host?
- Is there a difference between B and T cell immunization and the production of enhancement?
- What substances form the antigens?
- Can enhancement be induced by components of lymphocytes rather than the intact functioning cell?

The purpose of this paper is not to attempt to answer these questions but to add some knowledge towards their solution.

The ultimate goal of transplant immunosuppression is to induce a specific state of symbiosis with the transplant and the host.[7]

REVIEW OF LITERATURE

GRAFT ENHANCEMENT

Immunological enhancement acquires its name from the early observations of Flexner and Jobling in 1907.[41] It was noted that preimmunization of an animal with nonviable tumor cells allowed the tumor challenge to grow and kill the animal which would otherwise have resisted the tumor challenge and survived. This observation was contrary to the thought that preimmunization of tumor cells would enhance tumor rejection. This is in contradistinction to the observation of Pasteur who noted prior immunization provided immunity from infection. The paradox remained that preimmunizing against a transplant is a way of preventing its rejection. This can be shown by demonstrating passive enhancement. For example, one can immunize an inbred animal of strain A using the cells obtained from a second strain B. This raises alloantiserum in animal A. Taking the serum from animal A and injecting it into a second animal of the same strain A_1 will allow A_1 to accept grafts from strain B without rejection. The passive enhancement, i.e. via the serum, is maintained as long as the alloantiserum from strain A is injected into A_1.[56]

In active enhancement the immunized animal itself is the recipient of the graft. For example, if a strain A rat receives a liver transplant from strain B rat, the rat will reject the strain B liver; however, if immunosuppression is used for a period of approximately three months, the immunosuppressive agents can be withdrawn and the liver frequently maintains its function and is not rejected. If a heart graft from strain B is

performed on this animal which has received the liver transplant, the heart is not rejected despite the lack of reinstituting immunosuppression for this second organ.

One would think that a mere analysis of the serum would reveal the active agent producing enhancement. Unfortunately, investigations into this area have not lead to any such agent. It is generally felt that the agents are likely proteins and may be expressions of the human leukocyte antigen (HLA) system, most probably antibodies.

There is a great deal of confusion in this particular area of immunology as evidenced by several reviews on this subject, most emphasizing the complexity of the phenomenon. Early work was performed without genetically defined animals; nomenclature was not standardized. Multiple immunologic manipulations were performed to produce an expected effect confusing the contribution of each to produce enhancement.[7,8,16,22,25,37,40,76,98,100]

The data related to tumor enhancement should be avoided because of the complexity of the neoplastic cell and its immunologic uniqueness. It is our interest to study normal tissues. The history of enhancement is a long one, and serious consideration of the method and content of transplants was greatly delayed, firstly, because the principle studies were confined to the realm of tumors, and secondly, skin grafts are particularly difficult to enhance compared with revascularized organ grafts.[82] Enhancement in transplantation was reviewed by Wassen in 1971. Work on vascularized organ transplants was not possible until genetically defined animals large enough for application of microsurgical methods became available. The initial work

was done on mice which were generally too small to reliably perform transplants of solid organs. Once inbred strains of rats were identified and readily available, enhancement was shown to be a significant method of immunosuppression.[105]

Rat strains with different MHC's (major histocompatibility complexes) demonstrate markedly different responses to histoincompatibilities. Thus grafts tend to be accepted quite readily in certain strains more than in others. Enhancement tends to be more dramatic between certain strains than others, and sometimes an experiment that works in compatible strains may not work in others. It is also noted that certain types of grafts rejected more rapidly than others, for example,those grafts that tend to be closest to the environment reject more readily, i.e. skin grafts tend to reject more rapidly than liver grafts. Furthermore high and low responsive genes are also a possibility (IR genes). The hierarchy for rejectibility of different tissue has long been recognized and is generally now ascribed in part to their content of dendritic cells, i.e. those passenger leukocytes and histocytes that respond to an antigenic challenge, and their class 1 MHC antigen density, i.e. the major histocompatibility complex expression antigens. Known MHC antigens and their position on the chromsome may also have an effect.[15]

There is often confusion arising between the definitions of active enhancement and immunological tolerance as discussed in the introduction. This confusion arises because both are induced by treatment of a potential transplant host with donor type antigen. In fact these phenomena are distinct. Tolerance can be induced by providing antigen in a tolerogenic

(nonimmunogenic) form. While enhancement requires immunogen, tolerance may be induced most easily in non-MHC incompatible partnerships by injection at the time of transplantation.[84] For the establishment of enhancement, however, the immunogen must be provided long enough in advance to allow an immune response to be elicted, i.e. one or two weeks before transplant. Thus tolerance induced across an MHC barrier is not easily produced and often requires an additional set of circumstances for it to be produced, such as a short course of immunosuppression.[12,53]

Enhancement was induced with humoral antibody. This production of humoral antibody is not without hazard. The serum may contain antigen antibody complexes where the antigen is immunogenic, exposing the patient to immunocomplex disease or sensitizing the individual, thus enhancing rejection rather than the graft. The complexities arise because of the number of variables in the clinical situation, such as various animal species, strains, pretreatment, tissue grafted, amount of material grafted, routes of injection, timing, antigen responses, passenger leukocytes, degree of trauma attached to surgical procedure and so on. The simplest concept is that intolerance antigen is provided and an antibody arises later by an immune response to this, or the graft, or both. Donor antigen and antidonor antibody are the result. In passive enhancement the antibody that provided antigen comes from the graft. There are two different types of tolerance: self tolerance, the example, used with two sequential grafts; and perinatal tolerance, as in dizygotic twins.[24]

In studying enhancement there is a parallel between enhancement and the effect of antileukocyte serum. Enhancement is a very specific alloeffect.

Antilymphocytic serum is a nonspecific xenoeffect. Antilymphocytic serum is made from antithymocyte globulin. Thymocytes are the best immunogen to generate antithymocyte globulin. The T cells are the actual observed target;[34] as opposed to B cells which are necessary for immunogenic enhancement.[20] It is of note that B cell antibody cross reacts with Ia antigens implying that if dendritic cells in the graft act as old passenger cells which are the target of class II antibody in the establishment of enhancement, the dendritic cells are not the foreign antigen but the presenter of foreign antigen class I.[95]

Enhancement experiments are usually carried out using hemizygous, i.e. F_1 donor, transplants because homozygous cardiac and renal grafts are rarely susceptible to enhancement in rats.[35] It seems that halving the strength of the incompatibility halves the power of rejection and brings a wider range of experimental models into play. It is interesting that the factor of immunization does not seem to change with the dose of immunogen, for example, in mixed lymphocyte reactions there is no additive effect when more or less MHC immunogen is present on a per cell basis.[89] Therefore this implies that either a humoral response is responsible and not a cellular one, or that its rejectibility is diminished. It is known that antibody complement group mediated cytotoxicity in vitro and halving the antigenic sites does indeed diminish the reaction by limiting the site and density.[89] The question of anti-idiotypic antibody is raised by this phenomenon, and the idiotype is explained in the following way. The expectation on immunizing F_1 hybrid rat with a parental one (P_1 one of the parental strains) is that no immunological response will be obtained as the F_1 has a dose of what the P_1 strain presents, summarized as the F_1 having codominant transplantation antigens from

the parent; however, the F_1 could make anti-idiotype antibody against P_1 receptors, this specificity being forbidden by self-tolerance in the F_1 animal itself. The serum from an F_1 animal, having developed these antibodies, can be injected into a second parental strain, say P_2. This will prolong the survival of a graft in P_2, having F_1 genetic make up.[21]

In rat cardiac enhancement models, although F_1 transplants are generally used, the cells used for preimmunization in either active or passive enhancement experiments are often P_1 cells. Sometimes F_1 cells are used, but in many cases both P_1 and F_1 cells are seen to be used as though they are interchangeable, thus F_1 cells are incapable of eliciting an anti-idiotype response, but P_1 cells could do so, thus confusing the issue of enhancement. It is known when using F_1 heterotopic cardiac grafts in P_1 rats in an active enhancement model (DA X PVG) F_1 into PVG that preimmunization a week before grafting using F_1 cells or using P_1 cells gives different results.[15] P_1 cell are capable of inducing anti-idiotype response and give better graft prolongation than a similar treatment using F_1 cells which are unable produce anti-idiotype response if immunized into an F_1 recipient, B cells possess class I and class II antibody whereas non-stimulated T cells contain class I only. Therefore theoretically if anti-idiotypic antibody was the sole means of graft prolongation one should see similar prolongation by using either cell or mixed cell immunizations pretransplant. The existing experimental evidence attributed to enhancement has been anti-idiotypic and has no doubt served to compound the confusion and contribute to misquoted data.[15] Some studies have attempted to classify the agent which produces enhancement.[30,89,106] The most likely candidates for enhancement are G immunoglobulin (IgG) and are able to produce cytotoxicity. There seems to be

agreement that immunoglobulin M (IgM) does not enhance and that complement binding capacity is not essential.[64] The fact that certain parents show more enhancement than others may be an effect of immunization schedules used in different studies, using different cells, different times, and routes, as well as different quantities. It has been noted, for example, that, in general skin, grafting gives a potent enhancing antiserum with low in vitro cytotoxicity.[55]

The difficulty in discovering the class of antibody should be able to be worked out now that monoclonal antibodies are available.

It is generally thought that antibody antigen interaction is highly specific.[109] The observation that monoclonal antibodies are not absolutely specific is a difficult concept to understand. One way of explaining this phenomenon is to postulate subloci on the MHC. The generic expressions, i.e. antigens, coexist with antibodies indistinguishable from those used in tissue typing. This concept was initially put forward by Medawar in 1958 in order to explain the observation that antigens on rodent red cells are not capable of eliciting an alloimmune response (as measured by hastened skin graft rejection) while antigens on leukocytes are an adequate sensitizing stimulus by the same definition. The proposition of H-antigens and T-antigens arose.[9] A similar distinction could be made in the capacity to stimulate mixed lymphocyte reactions, and with this in mind a putative new gene product was sought by absorbing a mouse alloantiserum to completion with red cells and testing the absorbed sera for new antibody. These absorbed sera retain cytotoxicity only for certain restricted target cells, and in vivo the sera had undiminished capacity for enhancement having no H_2 antibodies, i.e. the

known histocapability antibodies at that time.[19] These enhancement experiments used heterotopic cardiac grafts in the rat (Wag x Argus) F_1 to Argus combination. Normal rejection time was consistently 7-9 days, but the original serum and absorbed serum gave permanent survival. Absorption with donor strain leukocytes (but not recipient strain leukocytes) removes all enhancing activity; thus old H_2 tissue typing (SD antibody now called MHC class I antibody) seemed to be at least unnecessary for enhancement and that new nomenclature Locus D (LD) equalling Ia in the mouse or Locus D sublocus r (Dr) in the human (now called MHC class II antibody) was the mediator.[17] The possibility that class I antigen antibody complexes may have eluted from the absorbing cells and played some role in the process was considered unlikely from the data presented at the time but will be reconsidered below.

Other non-mixed lymphocyte reaction (MLR) stimulating material could be employed for absorption in place of red cells, i.e. platelets.[11,22] These have been widely used for preparing class II antisera.[3] In man and primates generally red cells do not carry class II MHC antigens. Enhancement by class II antibody (B cell antibody) has been generally confirmed in a variety of models, especially for rat heart and kidney grafts.[93,107] Consistent with this data Meo et al showed that class II (Ia) antisera inhibited the stimulator cell in MLR, detracting from the clarity of the situation.[75] There are experimental models where class I antibody will enhance.[38,45,95,96,97] These exceptions may be understood in the light of an explanation of enhancement in the simple situation of induction of class II antibodies. It can be postulated therefore that stimulation by platelets

which do not carry class II MHC antigens could act like pure T cell enhancement without the risk of sensitizing the donor by stimulating class II antibody, hence, this would have some clinical application. Tarasaki has shown that red blood cells stimulate enhancement in renal transplants but the cost of this is sensitization of some individuals.[101] This sensitization is generally thought to be antibodies against class II antigen, and could be avoided if a similar effect could be obtained by platelets which obviously could not induce class II antibody and still produce the enhancing effect. Further experiments throw further light on certain class I and class II antibody reactions.[60] Class II antibody is thought to be a mediator of enhancement using cardiac allografts in the rat system (Wag x Argus) F_1 to Argus recipients in active enhancement. The enhancing capacities of B cells and T cells as preimmunizing antigens were compared. T cells judged to induce only class I antibody did not lead to enhancement. B cells judged to induce class I antibody and class II antibody did lead to enhancement. Further confirmation was obtained in another strain combination (DA x PVG) F_1, two PVG recipients. In this case B cell preimmunization gave enhancement but T cell preimmunization failed to produce enhancement. There are several kinds of experiments and clinical data to show the toxicity for class I antibody but none for any toxicity for class II antibody; thus, by continuing induction of IgG or subclasses it may be that it is necessary to enhance class II antibody or decrease class I antibody.[37,78]

Two phases of enhancement have been widely accepted - an induction phase and a maintenance phase.[8] The circulating antigen, antibody, or antigen-antibody complexes are presumably excess to requirements in the context of donor specific immunosupression, since only by interaction with

some cells is the potential activity to be realized. A component not detectable in the serum may be present only in sufficient amount to block this receptor.[39] Because class II alloantibody can initiate enhancement, it is an attractive proposition that the class II MHC antigen to which it attaches is that on donor dendritic cells, cells which are now believed to play a major role in initiating the alloimmune response.[48] They are very rich in class II (Ia, Dr) antigen. Such a mechanism for the initiation of enhancement by minimizing the initial sensitization fits well with the observation that small amounts of serum sometimes is sufficient for enhancement. This is an amount too small to cover up graft antigen in the sense of masking. Free graft antigen (class I) however is certainly exposed on cells of enhancing transplants. The evidence shows hastened rejection by class I antibody and that it is really class II antibody reacting with dendritic cells which initiate enhancement by preventing the alloimmune response from getting underway.[72,78] There is evidence that class I complexes directly block B cell differentiation to plasma cells.[102] It is also clearly shown that complexes lead to differentiation of antigen reactive cells, and further, that this activity is real in passively enhanced skin allografts in mice.[70] There were no antigen reactive cells in mice carrying skin grafts as a result of having been made tolerant neonately. We know that the liver provides abundant class I antigen and little class II antigen and is a large amount of tissue as a transplant,[53] and least susceptible to rejection. Skin has abundant class II and little class I antigen,[59] and is most difficult to enhance into a maintenance phase. When serum or lymph from liver transplanted rats is transfered to new untreated syngenetic animals which have been skin grafted from the same donor the graft is preserved but only while serum injections are maintained; after treatment this stops.[18]

Skin graft rejection then begins to take its normal course as though the treatment could provide for a maintenance phase but not carry out the initiation process. More specifically, perhaps, the class I complexes in the liver grafted rat serum did not react with skin graft dendritic cells (Langerhans' cells) effectively and class II antibody did not inactivate them under the conditions of the experiment, although there are some experimental situations, especially in rodents, where immunosuppression can eventually be withdrawn without losing the graft even when the MHC barrier is involved. This is not on record in the clinical practice.[103] Long surviving renal transplants with excellent function in patients on low levels of immunosuppressive drugs have rejection episodes if treatment is withdrawn. This kind of adaptation is not complete and its basis, though much discussed, is not known. One might ask whether enhancement enters into this and into some kinds of satisfactory clinical transplant situations, including those resulting from blood transfusions. No answer can be given because the end result of an enhanced situation still cannot be precisely described in terms of resulting B cell and T cell functional modifications. Rather little effort has been made so far to test possibilities of using enhancement clinically.[43] Some clinical work has been done in the area of antibody enhancement. An attempt was made to study this by the use of pooled B cell antibody administered to renal transplant patients.[88] In this trial blood donor volunteers were cross-immunized and the resulting antisera pooled and absorbed with platelets to remove the cytotoxic class I antibody. The pool size was judged adequate from the known cross reactivity of Ia specificities, unlike that of class I (tissue typing) specificities; but, in retrospect, the amount of class II antibody in the absorbed sera was probably insufficient to be influential and the final outcome was inconclusive. Studies have been made in the lower primates with similar results.[92]

In enhancement both B and T cells are modulated but IgM antibody against donor MHC can be made, and T cells are still able to react in MLR in graft versus host reactions. It is important to remember that many organs in many MHC combinations cannot be enhanced, and favourable experimental data come from the combinations purposely chosen to study the effect where it works best. Background immunosuppression with drugs will always be needed, and the studies of what other methods do and do not synergize with enhancement are important.[76]

In summary the minimal mechanisms to satisfy the simplest situation seem to require a means of neutralizing (killing) dendritic (antigen presenting, MHC class II positive) cells in a graft to prevent the alloimmune response from building up, i.e. an initiation phase, and this often can be maintained by class I antibody mediated antigen reactive cell opsonization and class I complex mediated inhibition of B cell maturation. These do not always work.[54] Thus, enhancement may be different in degree, and its principle will apply according to the particular tissue transplanted, power of the initiator, and the vulnerability of the target.

MATERIALS AND METHODS

EXPERIMENTAL MODELS: RABBIT KIDNEY ALLOGRAFTS, RAT HEART ALLOGRAFTS, RAT HEART AND LUNG ALLOGRAFTS. REVIEW OF 464 CARDIAC ALLOGRAFTS

Introduction

In order to study the effects of preimmunization on enhancement in transplant rejection, a suitable animal model had to be developed. Requirements were:

(a) Ready availability of large numbers of animals.
(b) Non-elaborate technique for transplanting a vascularized graft.
(c) Low mortality, high success rate.
(d) Easily-defined end-point of graft rejection.
(e) Low variance of end-point of rejection, reducing numbers of animals necessary in each experimental group, implying the use of reliably inbred animal strains.

A. Rabbit Kidney Allografts

Initially rabbit kidney allografts were studied. Kidneys were transplanted from New Zealand white rabbits to non-New Zealand white rabbits of a hybrid strain, using the technique of Dunn.[31] Young adult rabbits weighing 2-3 kg were used. Kidneys were transplanted orthotopically and recipients were bilaterally nephrectomized at the time of transplantation. Serum creatinine was measured every two to three days.

The rabbit renal allograft model was found to be unsuitable for the planned experiments, for the following reasons:

(a) Although the animals were readily available they are relatively expensive to purchase and maintain.

(b) The technique is simple, using a cuff technique for the arterial anastomosis, but the perioperative mortality was high. Even in isografts, the survival rate was less than 50% at 14 days. Death was due to several factors including pneumonia, hypothermia, renal arterial and venous thrombosis, and ureteric blockage.

(c) The end-point of rejection was not well defined. Serum creatinine may rise because an animal is unwell (for example developing pneumonia) rather than rejecting the transplanted kidney. Death may be due to causes unrelated to rejection, yet histology of an allografted kidney will still show histological changes of rejection.

(d) The end-point of rejection, being difficult to define, had a high variance.

(e) It was not possible to devise a system for continuous intravenous infusion in rabbits, without close confinement of the rabbits. The rabbits were able to destroy all cannulae by biting them, or by spinning rapidly around in their cages. For these reasons, attention was directed towards a rat heart allograft model.

B. Rat Cardiac Allografts

The rat heterotopic heart graft technique, modified after Heron[51] was used. In this system hearts are transplanted to the necks of recipient rats. Their subcutaneous location makes them particularly easy to assess for evidence of rejection: cessation of palpable beat is usually taken as the end-point of rejection. This procedure was found to be very suitable for further planned experiments, and the procedure became the standard experimental model for a large proportion of the work described in this thesis.

In the following section, the procedure used for heterotopic transplantation of rat hearts is described, and a general review of the first 464 transplants performed by the author is made. All the requirements for a suitable model for further experimentation were satisfied:

(a) Animals were readily available in large numbers. DA($RT1^a$)[1] and PVG($RT1^c$) rats were bred by the author from stocks obtained from Bantin and Kingman, Grimston, Hull. Regular syngeneic and allogeneic skin and heart grafts confirmed consistent patterns of graft acceptance or rejection in untreated rats, with no evidence of outbreeding.

(b) The transplant technique was simple, and rapidly learnt.

(c) The operative mortality was low, and the overall success rate, including death due to pneumonia and graft loss due to arterial or venous thrombosis was 78.5%.

(d) The end point of graft rejection, taken as cessation of palpable heart beat was well-defined, and occurred within one or two hours.

(e) The variance of end-point of rejection in control allografts was low. In both male and non-pregnant female PVG($RT1^c$) rats receiving hearts from DA($RT1^a$) rats of the same sex, the mean rejection time in untreated rats was 7.38 ± 0.77 (SD) days.

i. Technique for heterotopic transplantation of rat hearts

The method used was that of Heron[51] with certain modifications. Hearts were removed after ligating all vessels except for the aorta and pulmonary artery, and the stumps of these vessels were joined to the recipient carotid artery and jugular vein, respectively, using cuff techniques (Figure 1). Arterial blood therefore perfused the coronary circulation, drained to the right side of the heart, and was ejected from the graft pulmonary artery into the recipient venous circulation. The graft beat was vigorous, and, in allografts, cessation of palpable graft beat was taken as the end-point of rejection. This was a definite end-point, and in untreated allografts between inbred species the rejection time had a low variance.

ii. Materials and methods

Instruments For the technique to be described, the following instruments were used. Most were obtained from John Weiss and Son Ltd., 11 Wigmore Street, London W1H ODN.

Number	Catalogue Number (Weiss)	Instrument
1	BC 54	Dissecting forceps
1	BC 100	Straight Mayo scissors
1	BC 37	McIndoe forceps
1	B 1047	Sharp straight scissors
1	-	Large arterial clamp
2	B 981 C	Curved Mosquito forceps
1	B 1050	Sharp curved scissors
1	-	Microsurgical coagulator
1	B 1201	Magnifying loupe
1	BC 30	Curved jeweller's forceps
1	BC 31 5AR	Straight jeweller's forceps, deflected tip
2	-	Microsurgical vessel occluders
1	C 14	Microsurgical needle holder
1	B 981	Straight Mosquito forceps

iii. Other materials

Cork mat

Round headed pins

6 cm cube of modelling clay

10 ml syringe

19 gauge needle, point sawn off and edge rounded

Portex cannula, 3 F.G. O.D. 1.02 mm (for arterial cuff)

Portex cannula, 5 F.G. O.D. 1.65 mm (for venous cuff)

Razor blade

5'0' black silk -

6'0' black silk

8'0' Ethicon suture (Ethicon no. W2808)

4'0' catgut suture

Sodium chloride injection B.P.

Heparin

Ether

10 ml syringe container

Cotton wool

Hypnor* (Janssen)

Atropine

Cotton wool buds

iv. Procedure: Donor Operation

Twenty-five (25) ml of saline were transferred to a small plastic pot surrounded by ice, and 250 u of heparin added. This was for perfusion and storage of the heart to be removed.

A donor rat weighing 140 to 300 gm was anaesthetized in an ether jar until respiration started to slow. The rat was then placed supine on the cork board, using round headed pins through the web-skin of the feet to secure it. Anaesthesia was maintained by placing the 10 ml syringe container containing cotton-wool moistened with ether over the nose to the level of the incisor teeth.

A midline abdominal incision was made using forceps and straight Mayo scissors. The small bowel was displaced upwards and 250 u heparin injected into the inferior vena cava using a 25-gauge needle. The xiphoid was lifted with forceps and an incision made with scissors around the right border of the diaphragm. The ribs were cut posteriorly, parallel to the thoracic spine on either side. A large arterial clamp was attached to the xiphoid, and the whole anterior thoracic cage lifted superiorly while the pericardium was opened. The heart was cooled with topical saline at 4°C. Using forceps and straight scissors the inferior vena cava was mobilized, and then the right superior vena cava. These vessels were then ligated with 5/0 silk: one end of each ligature was left 2 cm long.

The tip of a curved Mosquito forceps was placed in the coronary sinus from the left to the right; the aorta was mobilized and divided across just below the first branch. Ten (10) ml of heparinized saline at 4°C were carefully injected into the aortic stump, with gentle closure of the open end of tne aorta with McIndoe forceps. The pulmonary artery was mobilized and divided just proximal to its bifurcation with sharp curved scissors. A very small vessel at the aortic root was coagulated. The superior vena cava and inferior vena cava were divided distal to the ligatures. The heart was then pulled anteriorly and a 5/0 silk mass ligature applied around the tissues posterior to the atria (mainly pulmonary veins and left superior vena cava). The heart was freed by dividing the tissues distal to the mass ligature, and the heart placed in 4°C, to remain there while the recipient was prepared.

v. Recipient operation

A recipient rat weighing 150 to 350 gm was injected intramuscularly with 0.5 ml/kg of Hypnorm. Ten minutes later it was injected subcutaneously with 120 pg/kg of atropine and the right side of the neck shaved with clippers. The rat was pinned out supine onto the cork mat, and the upper incisor teeth hooked over a rubber band which extended the neck.

A vertical incision was made slightly to the right of the midline from 0.5 cm below the first rib upwards to 0.5 cm below the mandible, using scissors and forceps. The lateral skin flap was mobilised with a

moist cotton-wool bud, and the flap pinned to the cork board. The fat overlying pectoralis major was lifted anteriorly and mobilized upwards using fine sharp scissors. One vessel penetrating pectoralis major was coagulated. When the jugular vein was approached this vessel was cleared anteriorly by blunt dissection to just above its main bifurcation. Using a magnifying loupe, and two pairs of jeweller's forceps for dissection, the jugular vein was mobilized: all branches except the bifurcation were mobilized and coagulated. The anterior branch was then ligated close to the main vessel, and then both branches were ligated together 0.5 cm. distal to the first ligature. The anterior branch was divided between the two ligatures.

The sternomastoid muscle was now mobilized, ligated with 5/0 silk as high and low as possible, and the central segment excised: this gap was used for the transplanted heart to rest in. The sternohyoid muscle was now teased apart in the middle of its belly, and the common carotid artery identified. Using jeweller's forceps the common carotid artery was mobilized from the vagus nerve, and the artery was then ligated just proximal to its bifurcation with 5/0 silk. A microsurgical vessel occluder was placed across the common carotid artery as proximally as possible, and then the vessel was partially divided just proximal to the ligature. An 8/0 Ethicon suture was used to transfix the left hand edge of the carotid artery, and the division of the artery completed. The jugular vein was also occluded with a microsurgical clip, and the posterior branch divided flush with the bifurcation of the main vein. Using a straight Mosquito forceps mounted on a block of modelling clay, a small cuff made from the 5 F.G. Portex cannula was mounted in the tip

of the forceps, and positioned over the jugular vein. The jugular vein was threaded through the lumen of the cuff and everted over it, and held there with a circumferential 5/0 black silk ligature. The cuff was separated from the Mosquito clip, and the process repeated with the carotid artery and a 3 F.G. Portex cuff, and a 6/0 black silk ligature.

The heart graft was removed from its saline bath, and positioned upside down in the neck. The aortic stump was placed over the carotid artery cuff, and held there with a 5/0 black silk ligature. The pulmonary artery was placed over the jugular vein cuff, and held there with a 5/0 black silk ligature. The venous and then arterial occluding clips were removed, and the graft perfused with blood. The graft started to beat immediately, or after a few seconds of ventricular fibrillation. The skin was closed with continuous 4/0 catgut, and the rat allowed to recover.

vi. Assessment of rejection

At the time of cessation of palpable heart beat the rat was anaesthetised with ether and the heart exposed. Occasionally, a serous collection was discharged, and the underlying heart was then seen to beat. More usually, cessation of beat was confirmed and the vessels were examined for evidence of venous or arterial thrombosis. In equivocal cases histology was used to differentiate between venous or arterial thrombosis or rejection. Initially, electrocardiography was used, but loss of palpable beat always coincided with loss of electrical activity (Figure 2), and direct examination of the heart was therefore sufficient.

For successful grafts using this technique, the following points are very important:

(a) Donors weighing over 300 gm are unsuitable as the heart is too large, and easily flops over in the neck causing venous congestion.
(b) Tissues must be kept moist and handled gently.
(c) Blood loss must be avoided.
(d) The jugular vein and carotid artery should be handled by contact with branches or adventitia wherever possible.
(e) Positioning of the heart must avoid pressure by the atria upon the pulmonary vein.
(f) The atria must not be distended at the time of closure of the skin: this is a sign of excess venous occlusion.
(g) The rat's nose should be kept away from sawdust during recovery, otherwise inhalation of sawdust and asphyxia is common.

C. Review of 464 Rat Heart Allografts

The results of the first 464 rat heart allografts performed by the author were analyzed. 97.4% (452 grafts) were initially successful, the grafts beating well at six hours (Figure 6). The 12 operative failures were due to shredding of the carotid artery during preparation of the everted cuff (3 grafts), heamorrhage from the venous anastomosis (6 grafts), uncorrectable venous congestion (2 grafts), or omission of heparin in perfusion fluid (1 graft). Venous thrombosis occurred in 5.5% (26 grafts) and arterial thrombosis in 3.5% (16 grafts). The total mechanical failure rate was 11.6%.

Pneumonia developed in 9.9% of recipients (46 cases) and this always led to death of the rats.

The total failure rate (or exclusion rate from experimental groups) was 21.5%.

Venous thrombosis occurred most often on day 1 or 2 (Figure 3) while arterial thrombosis occurred mainly on days 1, 4 and 5 (Figure 4). Pneumonia was most common from day 2 to day 6 (Figure 5), and was more likely when recipients weighing less than 200 gm were used. These results compare favourably with those of Heron who described a mechanical failure rate of 15% and a 10% incidence of arterial thrombosis.

The second series (Table 1) shows the results of the first experimental model as opposed to the overall results. The overall mortality was 26.6% in the first series.

D. Rat Heart and Lung Allografts

A technique for heterotopic heart and lung allograft transplantation in rats was developed. In the donor operation, both superior vena cavae were ligated and divided, and the inferior vena cava was cut flush with the right atrium. The aorta was divided and the heart and lungs perfused with heparinized saline. The trachea was divided high in the neck, and the heart and lungs removed together, and stored in cooled saline.

In the recipient operation, carotid and jugular cuffs were prepared as in the heart allograft model. The graft aorta was joined to the recipient carotid artery, and the graft right atrium was tied over the recipient jugular vein cuff.

When the vascular clamps were released a rapid return to sinus rhythm occurred, with good perfusion of the heart and lungs. The pattern of contraction of the heart appeared to be much more normal than that seen in heart allografts, probably because of minimal atrial disturbance. The trachea was brought out to open in the centre of the neck wound, and the lungs were periodically inflated using a syringe.

Although the initial results were good, all 10 recipients died within 76 hours due to respiratory distress: there was not enough room in the neck for the heart and two lungs to lie satisfactorily without causing venous congestion and tracheal compression in the recipient. This technique was therefore abandoned.

TECHNIQUE FOR B AND T CELL SEPARATION

The B and T cells were separated using ficoll-isopaque density gradients as described by Loos and Roos.[65]

A. Preparation of nylon column

It is known that B cells adhere to fine nylon strands due to their pseudopods, where T cells do not. This physiological difference between B and T cells is used to separate a mixture of B and T cells and null cells. For our purposes, 600 mg of nylon wool is used. This is packed into a 10 ml syringe and soaked with medium which is made up of a phosphate buffered saline and fetal calf serum. The following is the preparation formula for this mixture: sodium chlorate 8.0 gm, sodium hydrogen phosphate 1.15 gm, potassium chloride 0.2 gm, and potassium dihydrogen phosphate 0.2 gm. This is mixed with 1 liter of double distilled water. Then 0.1 gm of magnesium chloride and 0.1 gm of calcium chloride hydrous x 6 is added, and to this mixture is added 2.5 ml of fetal calf serum to each 100 ml of PBS (phosphate buffered saline). The medium for incubation contains 10% of fetal calf serum (FCS). The medium is allowed to soak through the nylon wool, and is incubated at 37°C until the cells have been prepared. The extra medium that is left over from the incubation is kept at 37°C as well, as is the PBS, FCS 2.5% mixture.

B. Preparation of lymph node or spleen cells

A similar procedure is done when either lymph node or spleen cells are used, i.e. the same steps are carried out. Technique described by Eremin, Plemb and Coombs, 1976.[33]

The lymph nodes or spleens are disrupted with fine scissors and scalpel in a mixture of PBS FCS. This is poured into universal Vortex tubes to let the larger lumps settle out for a period of two minutes. This suspension is decanted into fresh universal vessels. The mixture is centrifuged at 1600 revolutions per minute (rpm) for 10 minutes. The supernate is decanted. The sediment is mixed and filled again with PBS FCS. The larger clumps are allowed to settle and decanted again into equal tubes and filled to 10 ml. Using a syringe and slow injection, ficoll-isopaque is injected slowly until about 7-10 ml is filled. The cell suspension then rises above this much more dense medium, and this acts as a gradient, allowing heavier sediment debris to pass through, but not allowing lymphocytes to pass through, thus forming an interphase containing the lymphocytes. The sediment of white cells and erythrocytes tends to pass through and is caught in the vortex of the universal. The suspension is mixed with PBS FCS and centrifuged again at 1800 rpm for 10 minutes to wash out the excess ficoll. The supernate is then decanted and filled again with PBS FCS, and centrifuged once again at 1500 rpm for 5 minutes. The supernate is again decanted and mixed, and 5 ml of medium with 10% FCS is mixed. The mixture is then counted in a Neubauer chamber which is a counting chamber using gradations, and allows counting of the number of cells in a specific

volume. Finally, the mixture is put into an Eppendorf tube so that the cell concentration is between 50 and 100 million, in 2 ml of medium plus FCS 10%. This is suitable for IgG labelling. Now that a lymphocyte mixture has been obtained, separation between the T and B cell types is required

C. Separation of the B and T Cells (After G. Brons, Ph.D. Thesis in progress, University of Cambridge, U.K.)

The nylon column is washed with 5 ml of warm medium plus 10% FCS. The wool mixture is compressed slightly with the plunger of the syringe to pack the wool so all gross fluid is expelled, thus allowing maximum exposure to the surface of the nylon wool. The tip of the syringe is plugged, and dropwise, 2 ml of cell suspension is allowed to fall into the middle of the nylon wool syringe, and the cells are allowed to percolate through the nylon wool mixture. One ml of medium is added to the tube, and the remaining cells are mixed. A further 1 ml is added to the top of the nylon wool. This mixture of cells and medium is allowed to incubate for one hour at 37°C. The top of the tube is sealed with parafilm for airtightness and to prevent contamination by bacteria. The column is then opened at the bottom over the universal tube. Warm (37°C) PBS FCS is poured through the column into the Universal. A total of 25 ml is required, so that all nonadhered cells are washed through. The nonadhered cells are the T cells. One hundred (100) ml of warm PBS FCS mixture is percolated through the nylon wool and discarded. This removes the null cells, i.e. the non B, non T cells.

Ten (10) ml of warm PBS and FCS 2.5% is added to the syringe, and the plunger is applied to the remaining nylon wool, and the nylon wool is compressed with moderate force to expel the adherent cells. These are the B cells. This procedure is done twice to remove all the B cells. The cells, once labelled, are centrifuged at 1600 RPM for 10 minutes. The supernate is discarded, the sediment is again mixed, and added to 5 ml of PBS FCS. The cells are then counted, and the concentrations adjusted to 50 million cells in 1/2 ml. These cells are used for immunizations, while some are kept for T and B cell fluorescent assays. Note fluorescent assays in Figure 12; **summary of procedure,** Figure 9.

STAINING OF CELLS

Cells are stained to demonstrate that we were, indeed, separating the B from T cells, and that we were producing a low contamination rate of Null cells (See Figures 10,11 and explanation below). The cells are stained by two methods: 1) A peroxidase stain and 2) A fluorescein stain, both direct and indirect method.

Peroxidase Stain (after Heyderman[52])

Direct Method

Cells were fixed on the slide with methanol for 10 minutes. To eliminate endogenous peroxidase, the cells were washed in a 2:1 solution of methanol and hydrogen peroxide for 10 minutes then cleared with a saline wash. The

cells are then stained with anti-rat IgG peroxidase diluted 1:10 for 30 minutes at room temperature (Miles Peroxidase Anti-Rat IgG No. 61-206, specific for rat B cell surface antigen). The slide is washed with saline three times after counter staining with Carrazi stain.

Fluorescein Stain

Direct Method

The same process was used as for the peroxidase stain except for the peroxidase wash. Miles FITC Anti-Rat IgG from the Rabbit No. 65-174 (specific for rat B cell surface antigen) was used for staining.

Indirect Method

The B cells were fixed on the slide with ethanol for 10 minutes, then washed with 2:1 methanol:water solution. The cells were incubated for 30 minutes at room temperature with Anti-rat IgG raised in rabbit used as the intermediate (Miles No. 65-160). Anti-rabbit FITC (fluorescein labeled) raised in the sheep was used as the antibody staining (Miles No. 65-173).

PREPARATION OF FETAL CALF SERUM ABSORBED WITH SHEEP RED BLOOD CELLS

Five cc of sheep's blood freshly taken is spun 250 rpm for eight minutes. The supernate and buffy coat are both pipetted off. PBS is added and mixed. This mixture is again centrifuged at 250 rpm for eight minutes. This process is repeated once more. The tube of fetal calf serum is heated and activated at 56°C for 30 minutes. The sheep red blood cells are then mixed with the fetal calf serum and mixed again and incubated at 37°C for 30 minutes, then placed at 4°C overnight. Once done the mixture is centrifuged at 3000 rmp for 15 minutes. The supernate is the absorbed fetal calf serum and is placed in 2 ml aliquots for future use (0.2 gm of KCl, 0.2 gm K-orthophosphate KH_2PO_4). This is added to fulfil a volume of 1 litre of double distilled water. It is mixed for five minutes. Under a laminar flow system 0.1 mg of magnesium chloride and 0.1 mg of anhydrous calcium chloride are added to this litre. This is kept at 4°C and is changed once a week.

STATISTICAL ANALYSIS OF HEART TRANSPLANTS IN RATS

Statistical analysis was performed by Michael Butts, East Anglia Cambridge Medical Health District.

The mean survival of the PVG heart into a DA recipient was 6.8 days. The mean survival of a (DVG x DA) F_1 heart transplant into a DA recipient was 8.6 days. These two groups are significantly different $p<0.05$. This difference is expected as F_1 grafts have half the genetic disparity and therefore take slightly longer to reject (Table 2).

DA rats immunized with non-heated T cells derived from PVG rats produced no difference in graft survival from PVG controls after F_1 hearts are transplanted. If (PVG x DA) F_1, T cells are used, the mean is the same despite the greater mean in the F_1 control groups. Despite this difference there is no significant difference between the two groups (Table 3).

When B cells are substituted for T cells there is a signficant difference between non-heated B cells and controls. This difference is maintained despite the use of PVG B cells or F_1 B cells (Table 3).

When T cells heated to 56°C were used to immunize the recipient the mean graft survival for the PVG T cell group was 5.9 days and for F_1 T cells was 6.4 days, again not different from controls (Table 4). If B cells heated to 56°C were substituted for the T cells immunizations the mean survivals for PVG B cells was 13.8 days and for F_1 B cells was 9.8 days. There is a significant difference between the PVG groups but not the F_1 group (Table 4).

Comparing the non-heated B cells to the 56°C heated B cell immunized group, there is no difference between these groups.

When PVG or F_1 T cells were heated to 85°C and immunized into DA recipients no difference was observed. When PVG B cells were substituted for T cells the mean survival of the graft was 19.7 days, significantly longer than nontreated heart recipients. Comparing this with the non-heated B cells there is no difference in survival but there is a difference when 85°C heated B cells are compared to the 56°C heated B cells (Tables 3,4,5).

When platelets were purified and immunized prior to heart transplant, no significant prolongation was obtained.

Because of the complexity of the groups and the number of variables being compared, a multivaried analysis was carried out. The variables were: 12 experimental groups, each with a certain number of members. Variables included PVG cells, F_1 cells. Of these specific groups of cells, they could either be T cells or B cells, and a further sub group could be unheated, heated to 56°C, or heated to 85°C. Noted in an analysis of one variable, all main effects significantly affect graft survival. In addition, the type of rat and the type of cell affects survival. If a star is noted by the figure, this is significantly different ($p<0.05$). If two stars are noted by the number, it is within confidence limits ($p<0.01$).

An important observation noted in Table 5 is that long term survivals were obtained by immunization with 85°C heated PVG B cells. It appears to show that some of the transplants are in fact influenced differently by the immunization thus producing a subgroup which cannot be compared as a whole. If this is true then assigning Rank Sum comparision statistical methods does not apply.

STATISTICS

NUMBER OF VARIABLE	1	
NUMBER OF CASES	9999	
NUMBER OF LEVELS IN A	2	
NUMBER OF LEVELS IN B	2	
NUMBER OF LEVELS IN C	3	
NUMBER OF FORMAT CARDS	1	
DATA INPUT MODE	0	0 = CARDS / 1 = FILE
MISSING DATA MODE	0	0 = NONE / 1 = MISSING DATA

9999 CASES IMPLIES THAT UNEQUAL GROUPS ARE INPUT

DATA FORMAT IS (I0.F0.0)

GROUP 1	(9 MEMBERS)	PVG, T, UNHEATED
GROUP 2	(7 MEMBERS)	PVG, T, 56
GROUP 3	(8 MEMBERS)	PVG, T, 85
GROUP 4	(12 MEMBERS)	PVG, B, UNHEATED
GROUP 5	(6 MEMBERS)	PVG, B, 56
GROUP 6	(8 MEMBERS)	PVG, B, 85
GROUP 7	(9 MEMBERS)	F1, T, UNHEATED
GROUP 8	(8 MEMBERS)	F1, T, 56
GROUP 9	(8 MEMBERS)	F1, T, 85
GROUP 10	(10 MEMBERS)	F1, B, UNHEATED
GROUP 11	(10 MEMBERS)	F1, B, 56
GROUP 12	(6 MEMBERS)	F1, B, 85

ANALYSIS OF VARIANCE

SOURCE	M.S.	D.F	F-RATIO	PROBABILITY
TOTAL	34.589	100.		
BETWEEN	215.852	11.		
A = PVG/F1	103.232	1.	7.7565	0.0066**
B = T/B	779.840	1.	133.7311	0.0000**
C = UNHEATED/56/85	122.377	2.	9.1950	0.0004**
AB	119.957	1.	9.0132	0.0038**
AC	1.453	2.	0.1091	0.8964
BC	59.560	2.	4.4752	0.0139*
ABC	2.281	2.	0.1714	0.8438
WITHIN	13.309	89.		

MEANS FOR ALL EFFECTS

```
**************************************************************************

A MN              1 (PVG)           2 (F1)
                 12.0817           10.0204

SPECIFIC COMPARISONS (TUKEY METHOD) FOR ALL CELLS

CELL A 1
       A 2   4.02**
**************************************************************************

B MN              1 (T)             2 (B)
                  6.7715           15.3306

SPECIFIC COMPARISONS (TUKEY METHOD) FOR ALL CELLS

CELL B 1
       B 2  -16.67**
**************************************************************************

C MN              1 (UNHEATED)      2 (56°)          3 (85°)
                 11.1986            9.0378         12.9167

SPECIFIC COMPARISONS (TUKEY METHOD) FOR ALL CELLS

CELL C 1
       C 2   3.41*
       C 3  -2.71
CELL C 2
       C 2  -6.12**
**************************************************************************

A.B               1 (T)             2 (B)
       1          6.6911           17.4722
       2          6.8519           13.1889

SPECIFIC COMPARISONS (TUKEY METHOD) FOR ALL CELLS

Cell A 1
     B 1           B1               B2
       A 1                        -14.84**
       A 2       -0.22             -8.95**

CELL A 2
     B 1           B1               B2
       A2                         -8.95**

CELL A 1
     B 2           B1               B2
       A 2       14.62**           5.90**
**************************************************************************
```

PURPOSE OF STUDY

In the following experiments, the assumption is made that enhancement is simple, by examining some supposed beliefs. Previous work, noted in the introduction, showed that class II (Ia) antibodies can mediate passive enhancement, and subsequently that active enhancement is mediated by preimmunization with B cells. We wish to show that true enhancement can be distinguished from prolongation due to anti-idiotypic antibodies. We also wish to find the role of class I allo-antibodies which may hasten rejection, and the role of class II antibodies which is likely confined to prolongation of graft survival.

EXPERIMENT 1

Are T cells able to provide the immunogen for enhancement? From the previously described work of Lauchart et al,[60,61] they may, after several immunizations with mixed cells, but do T cells demonstrate the same effect after a single immunization? B cells and T cells have identifiable surface antigens, and therefore can be separated out so that immunization can be carried out using pure T cells or pure B cells. By preimmunizing a recipient seven days prior to engraftment of a F_1 heart, one could observe the effect of either pure T cell or pure B cell pretransplant immunization.

PVG donor cells were obtained by:

Dissecting the lymph nodes, which are rich in T cells, and obtaining separated T cells by the method described in Cell Separation Technique

(Figure 9). The cells, thus separated, were injected intravenously into DA rats seven days prior to engraftment. The recipient animals weighed between 125 and 250 gms. A total of 1×10^7 cells were injected seven days prior to engraftment. The animals were kept in individual cages and observed for the seven days to be certain that they did not develop any sepsis or other ill effects secondary to the immunization. On Day 7, a (PVG x DA) F_1 heart was harvested by the method entitled, "Donor Operation". This heart was then transplanted into the recipient in a heterotopic location, thus not supporting circulation. (See Method entitled, "Recipient Operation")

These animals were observed daily until the donor heart rejection was noted to be the time at which the heartbeat ceased. The animal survives after the donor heart is rejected, and the heart generally involutes. Note Figure 7, Acute Allograft Rejection. The rejected heart was not removed, as the animal survived with the rejected heart in place, and after a period of time, the heart fibrosed down to a scarred remnant which did not affect the animal's overall wellbeing (Figure 8).

The results of this experiment, utilizing T cell preimmunization, are presented (Table 3; Graphs 1,2,3). The overall mean survival of these allografts was 6.5 days, which is shorter than the expected rate of survival in the control (PVG x DA) F_1 by DA which had a mean survival of 8.6 days (Table 2; Graph 4,5). An analogous experiment was carried out using B cells instead of T cells using the method just described. Instead of dissecting out lymph nodes, which are rich in T cells, PVG spleens were removed, and these cells were separated by the method of Loos,[65] thus obtaining

approximately 1 x 10^7 purified B cells, and immunizing them into the DA recipient seven days prior to engraftment with an F_1 heart. This time, the engrafted hearts survived a mean of 17.6 days (Table 3 - #2).

EXPERIMENT 2

Is anti-idiotype involved in this phenomenon? As mentioned in the introduction, enhancement is likely different from anti-idiotype prolongation. In order to demonstrate that anti-idiotype antibody production causes this observed phenomenon in Experiment 1, an analogous experiment was carried out, utilizing not PVG B cells or T cells, but (PVG x DA) F_1 T cells or B cells, and injecting them into DA recipients, and then engrafting an F_1 heart. If anti-idiotype were the sole mechanism responsible for enhancement one would expect that B cells and T cells would produce a similar response. If anti-idiotype antibody was dose dependent (i.e. F_1 transplants survive longer) PVG B or T cells should produce a shorter prolongation than F_1 B or T cells.

The results of the engraftment of F_1 hearts into DA animals previously immunized by (PVG x DA) F_1 T cells one week prior to engraftment, are shown on Table 3 - #3 (Graph 4). The mean survival was 6.5 days, similar to those previously immunized with PVG T cells (Line 1 compared to Line 3 - Table 3).

When (PVG x DA) F_1 B cells were immunized one week prior to engraftment in a DA animal with a (PVG x DA) F_1 heart, the mean survival was 14.1 days. This is significant and different from Line 2 - Table 3 (Graph 5).

EXPERIMENT 3

Characterization of the immunogen. Is it a protein or a carbohydrate?

Immunogens are noted to be proteins or mucopolysaccharides. The carbohydrate portion of mucopolysaccharides is also known to be immunogenic. A classic example of this is the capsule of certain viruses and bacteria. Carbohydrate capsules do produce antibody responses, and therefore are immunogenic. It is not known what the immunogen is in enhancement.[62,94,100] Having demonstrated that B and T cells have different immunization potential and that B cells do, in fact, prolong graft survival by a mechanism of non-idiotype active enhancement, it is important to characterize the initiator of this response. The cell membrane is known to be immunogenic, and contains antigens.[99] The cell wall is made up of both protein and carbohydrate moieties. The internal structures of the B or T cell contain the products of nuclear DNA and RNA production and are predominantly proteins.[85] To answer the question whether or not the immunogen is protein or carbohydrate, it is important to determine whether the intact cell is necessary to produce the same effect, or whether denatured cells also induce graft enhancement by active immunization. Cells have their membrane proteins denatured by incubation at 56°C for half an hour. This incubation does not disrupt the cell membrane, but the presentation (HLA product) proteins are denatured. Carbohydrates are not altered on the membrane after this incubation, nor are interior proteins.[90]

EXPERIMENT 3-1

(PVG x DA) F_1 hearts were transplanted into DA recipients after having the recipient immunized with PVG T cells separated and incubated at 56°C for 30 minutes. The results show a mean survival of 5.9 days (Table 4 - Line 1; Graph 7). This result is similar to grafts using mixed cells (Table 6), and shorter than PVG T cells which are non-heated. This difference is not statistically significant.

EXPERIMENT 3-2

An analogous procedure was carried out, utilizing (PVG x DA) F_1 hearts engrafted into DA recipients, following PVG B cell immunization. This time, the B cells were incubated at 56°C for 30 minutes. The mean survival following this immunization was 13.8 days. This is longer than expected, but not as long as the unheated B cells (Graph 6).

EXPERIMENT 3-3

(PVG x DA) F_1 hearts were transplanted into DA recipients. This time the recipient was previously immunized seven days prior to engraftment with F_1 T cells incubated at 56°C for 30 minutes. The mean survival following this experiment is 6.3 days (Graph 7). This is not significantly different from non-heated T-cells.

EXPERIMENT 3-4

(PVG x DA) F_1 hearts were engrafted into DA recipients previously immunized with F_1 B cells, incubated at 56°C for 30 minutes. The mean survival following this immunization was 9.8 days, as opposed to 14.1 days in the nonheated B cell immunization experiments (Table 4 - Line 4; Graph 8).

EXPERIMENT 4

Denatured Cell Immunization

Cells are denatured by incubating them for ten minutes at 85°C (Table 5). Proteins would be denatured and carbohydrates would still have their biochemical integrity maintained. The following experiments were performed to observe the effect of immunization with cells having had their proteins denatured and the carbohydrate moiety exposed in the analagous situation as described in Experiments 1, 2, and 3.[23,87] The cells are not disrupted by this process (Figure 13). Cells were heated at 85° for 30 minutes. The cells themselves maintained their shape but became crenated after the internal proteins were denatured.

EXPERIMENT 4-1

F_1 hearts were transplanted into DA recipients after being previously immunized one week prior to transplant with PVG T cells incubated at 85°C for 10 minutes. The result of this experiment show a mean survival of 7.4 days. This is longer than the nonheated and 56°C immunized cells (Table 5 - Line 1). This difference is not significant.

EXPERIMENT 4-2

F_1 hearts were transplanted into DA recipients. On this occasion, PVG B cells incubated at 85°C for 10 minutes were used as the immunizing agent. The mean graft survival was 19.7 days. This was not significantly longer than unheated controls or B cells heated to 56°C. These groups may not be comparable, however, as a subgroup demonstrating long term survival seems to exist thus rendering comparison invalid (Table 5 - Line 2).

EXPERIMENT 4-3

F_1 hearts were transplanted into DA immunized recipients, T cells having been incubated to 85° for 10 minutes. The mean survival was 8.1 days (Table 5 - Line 3). This result was not significantly different from controls.

EXPERIMENT 4-4

F_1 donor hearts were transplanted into DA recipients after being previously immunized seven days prior to transplant by F_1 B cells incubated at 85°C for 10 minutes. The mean survival was 15.7 days. This approaches but does not reach statistical difference from 56°C heated cell immunizations. These results are summarized on Table 5 (Line 4).

EXPERIMENT 5

Mixed Cell Immunization

To determine whether an effect other than the separation of the B and T cells is active in the Experiments 1-4, cells were separated by the techniques mentioned in Cell Separation Technique and then mixed together and injected as a mixed B and T cell immunization. This experiment acts as a control for the biochemical and physical separation technique.

F_1 hearts were transplanted into DA recipients following the recipient being immunized seven days prior to transplant by mixed T and B cells having previously been separated. The mean survival following these transplants were 6.8 days for F_1 mixed cell immunizations and 7.3 days for the PVG mixed cell immunizations (Table 6). There are no significant differences from controls.

EXPERIMENT 6

Platelet Immunizations

It is known that class II antigen is not present on platelets and therefore be a similar immunogen as purified red blood cells which do not carry class II antigen either.[11] If exposure of class I antigen without exposure to class II antigen could induce enhancement the chance of producing sensitizing antibodies would be lessened. If platelets produce an effect similar in magnitude to whole blood there would be a theoretical advantage in

substituting platelets for whole blood when immunizing patients prior to transplantation.

EXPERIMENT 6.1

Four experiments were carried out to see if platelets could produce the effect noted in the previous experiments using B cells.

(PVG x DA) F_1 donor hearts were transplanted into DA recipients. PVG platelets harvested from 10 cc of whole blood treated with EDTA and centrifuged at 1,000 revolutions per minute produced a buffy coat. This buffy coat was aspirated by micropipette and washed serially with 5 cc of saline and ultra-centrifuged at 10,000 rpm for 10 minutes. This buffy coat contains white blood cells and platelets mixed and is separated from the serum and red blood cells. The ultracentrifugation performed with heparinized saline at 10,000 rpm removes the white cells, and leaves the platelets behind. Microscopic analysis shows only one white cell per 10,000 platelets, thus the white cell transfusion effect should not be active in platelet immunizations. By micro-counting, approximately 100,000,000 platelets were injected at intervals prior to transplant, intervals being 21, 14, and 17 days pretransplant. The survival of the (PVG x DA) F_1 heart in the DA recipient, having been immunized with serial PVG platelets, showed a mean survival of 9.2 days (Table 7 - Line 1).

EXPERIMENT 6-2

A (PVG x DA) F_1 heart was transplanted into a DA rat using ultra-centrifuged and purified F_1 platelets. The mean survival from this experiment was 10.3 days. This experiment was carried out subsequently with F_1 platelets (heated to 56°C for 30 minutes). The survival mean was 8.8 days. This result is not different from nonheated platelets (Table 7 - Lines 2 and 4).

EXPERIMENT 6-3

As in 6-2 except PVG platelets were used to immunize the DA recipients by the same treatment schedule. The mean survival of the non-heated platelets was 9.2 days. When platelets were heated to 56°C for 10 minutes, the mean survival was 9.0 days. Neither group's survival was significantly different from controls (Table 7 - Lines 1-4).

EXPERIMENT 7

Specificity Controls

To demonstrate that this effect is not entirely a result of the particular strain combination used, or separation artifact, three experiments were carried out to demonstrate that the effect was secondary to the immunizations.

1. AO ($RT1^o$) hearts were transplanted to DA recipients and these rejected between seven and eight days. A total of four grafts were carried out.

EXPERIMENT 7 Continued

2. An AO heart was transplanted into a previously immunized DA rat, and three types of immunizations were carried out: F_1, T, and B cells. Again, the mean rejection was 7.5 days.

3. A DA rat was previously immunized by AO T or B cells, and transplanted with an F_1 heart, rejected in the same period of time, showing no species peculiarity. The results are summarized in Table 8.

All three groups showed no significant difference from controls.

DISCUSSION

The development of a suitable model for rejection to study an effect on the immunologic integrity of the recipient is not simple. In order to show statistically significant differences in subtle alterations of graft enhancement a large number of transplants is necessary and the success rate must be high or technical factors overshadow the immunologic effect. The procedure must be rapid enough so that warm ischaemic time does not become a significant variable. Lastly, the end point of the graft survival must be abrupt and easily measured. The results of the 464 rat heart allografts show that the model is most suitable for assessment of the desired immunologic manipulations. The success rate of 97.4% demonstrates the reliability of the model and absence of a significant technical component. The rat heart transplants all had very similar and short warm ischaemic times permitting this to be discounted as a variable. Separate experiments not reported here showed that a rat heart kept at 4°C for as long as four hours functioned after reperfusion. There is a definite learning curve as demonstrated by the complication rate in the first series of 96 rats compared to the overall results of 464 rat heart transplants (Table 1). Several modifications of the heart grafts technique were adopted after the pilot studies and learning phase were completed. These were mainly in the cuff technique of microvascular anastomosis which provided an intima to intima contact as well as the rapidity of completion thus decreasing ischaemic time and shortening the anesthetic (Figure 1).[51]

The end point of rejection is usually taken as that point at which the transplanted organ ceases to function or can no longer maintain the

homeostasis expected in the organ system of the recipient. For example, if a kidney is transplanted into a recipient the end point of rejection is that time when the animal dies of renal failure, but it could also be taken to be the point renal function has ceased, i.e. rapidly rising BUN or creatinine. The animal dies some time after the transplanted kidney has ceased to function and may be influenced by the metabolic rate as well as other variables, thus, the end point is not clear. The same can be said for pancreatic grafts. The end point is generally accepted as that point where exogenous insulin is required for the maintenance of glucose homeostasis. As hyperglycemia often is associated with stress due to infection, graft rejection or pancreatitis, the end point is often not clear.

The heterotopic rat heart transplant provides a reliable and accurate end point. It does not support circulation so the animal does not die at rejection. The time that the heart beat stops is taken as the end point and is the same point as isoelectric ECG tracings. The heart beat can easily be noted in its subcutaneous location in the neck as opposed to the abdomen.[63] Aortic pulsations may confuse the examiner (Figure 2).

Once the heart rejects, the organ is phagocytosed by the recipient leukocytes and disappears.

Isogenic heart grafts function for the life of the animal as do adequately tolerant immunosuppressed recipients.

Rat heart lung transplants were attempted but the results show that the technique is not suitable. The bulk of tissue was too large to be

transplanted in the neck and would seem to obscure any experimental effect produced.

It is clear that graft enhancement is a real phenomenon as evidenced by Tarasaki's work on pre-transplant blood transfusions.[57,58,101] It became evident that, in order to produce the desired enhancement, several blood transfusions were necessary.[5] This effect is diminished over time and, with the advent of cyclosporin A, this effect may be lost.[72] This evidence indicates that the ability to enhance grafts may not be a strong effect.[4] In order to reproduce enhancement a full HLA mismatch usually causes rapid rejection and enhancing is difficult. To allow a less rapid rejection F_1 donor organs are used, thus diluting the HLA incompatibility by a half.[26,27] The disadvantage of this model when applied to the clinical situation is that, apart from family, cadaver donors are not HLA matched.

PVG(RTI^c) and DA(RTI^a) have been shown to have a high immune incompatibility in previous transplant experiments using many strain combinations.[1,36] This strain incompatibility reduces the chances of a low response being mistaken for an effect of enhancement.[57]

The prolongation of grafts by pre-transplant immunization with B cells corroborated the effect shown by Lauchart in a different strain combination (WAG vs PVG).[60,61] Mixed lymphocytes failed to produce a similar effect. Although not significant, the tendency to hasten graft loss after T cell immunization is seem in some experiments. This may become signficant if the immunization schedule were to change as T cells tend to react with the host more rapidly producing more rapid rejection.[67,69] B cells are the source of

antibody production and pre-transplant stimulation of the B cell system may produce enhancing antibody. The response is speculative, but antibodies have been identified against killer T cells and antibody against antibody has been recognized.[44] A further theory postulates that the antigen presentation site in the donor may be masked by the enhancing antibody raised in the immunized host.[15]

A possible problem in using a F_1 (filial) donor heart is that anti-idiotype antibody will be produced when P_1 (parental) cell are used and to a lesser degree when F_1 B and T cells are used to immunize. No genetic differences exist between the sensitizing cells and the F_1 donor heart but anti-idiotype antibodies could be present in the DA recipient prior to engraftment. Anti-idiotypic antibody is likely not the sole cause of enhancement because of two observations: one, B and T cells both capable of producing anti-idiotype antibody have different survivals; two, no difference in survival was consistently noted when pure PVG or F_1 hybrid cells were used. Although F_1's are capable of producing anti-idiotypic antibody as are PVG, PVG would only partially protect the graft as anti-idiotypic antibody protecting PVG antigens would be produced.

It has generally been accepted that the immunogen is a protein product of the HLA transplant antigen system.[77] These antigens are found on the cell surface of B and T cells. Incubating the cell to 56°C for 30 minutes denatures these exposed proteins. If these antigens were critical in producing and enhancing antibody, destroying these proteins should reverse the prolongation observed in Experiment 1. This incubation did not disrupt the cell membrane integrity (Figure 13).

The incubated PVG B cells (56°C) still produced graft enhancement but not as long as the unheated B cell immunizations. The same observation was noted when (56°C) heated F_1 B cells were used.

Several conclusions could be made: (1) the denatured HLA protein is able to produce a partial response, (2) there are several mechanisms producing a net effect and heating to 56°C removed selected processes reducing the effect of enhancement, (3) the full effect could still be present but there is an alteration in the processing of the enhancing antigen by the recipient, (4) the process produces toxic material to the host interfering with the host's immune response.[68,69]

To address these problems one would be interested in seeing if the B cells which are heated are able to be immunolabelled to determine if the antigen is still intact. The heated B cells did, in fact, stain with both immunoperoxidase and antibody labelled with fluorescein, which shows that the immunologic presenting antigen is not entirely destroyed by heating to 56°C or 85°C (Figure 13). Thus, at least one presenting antigen complex on the B cell is not a pure protein but likely a protein carbohydrate rendering it heat stable, and some is protein being altered by the heating and decreasing enhancement once destroyed.

The T cells, whether PVG or F_1 did not seem to affect graft enhancement nor hasten its rejection. One could conclude from this observation class II antibody is necessary to produce enhancement or the immunization schedule was appropriated from the B cell antigen including class II but inappropriate for T cell antigens. One would need different immunization schedules using T cells to see if this is an important factor (Graph 7).[28,29,78]

The B cells heated to 56°C lost some of their enhancing effect but this effect seemed to return when heated to 85°C. This suggests that enhancement may be multifactorial and certain effects are lost while others may become unopposed. An important observation is that the enhancing effect is not lost when the cells are heated to 85°C suggesting a nonprotein moiety is able to produce enhancement. It is noteworthy that these long term survivals were generated by the 85°C B cell immunizations. This may be a subpopulation of animals who have responded to the antigen not destroyed by the heating. This observation is very encouraging, and we may be seeing a very powerful stimulus of graft enhancement.

These observations are donor species specific. This is demonstrated by immunizing the recipient of a heart graft with B or T cells from a different rat strain and does not produce graft enhancement. Furthermore, if the B and T cells, once separated are reconstituted and immunized, the enhancing effect of pure B cells is lost. This serves as evidence that the enhancement is not a biochemical artifact produced by the separation process or by B cell in general.

How have these experiments helped with clinical transplantation? One wishes to produce pure enhancement of the graft in the host without sensitizing the host in the process. Purified B cells will produce enhancement of heterotopically transplanted heart allografts in the rat model. Furthermore by heating the B cells to 85°C for 10 minutes, the B cells are not destroyed and the enhancing effects is not lost suggesting this effect is not produced solely by protein antigens. In the case of a living related (non-identical twin) donor, blood could be donated prior to

transplantation and the B cells could be separated and transfused instead of whole blood. There is no assurance that sensitization would be less likely to occur. In the above model MLR's could be performed comparing the sensitization potential of whole blood versus B cell immunization prior to transplant. Unfortunately, this type of immunization would likely be inconceivable for cadaver derived organs until preservation of the organs for much longer periods is developed.

In this particular model, platelet transfusion prior to engraftment did not have a significant enhancing effect. This does suggest that the class II antigen is important in the production of enhancement and is consistent with the observation that T cells do not produce enhancement.

Table 1: Model - Heterotopic Heart Transplant to Neck of Recipient First Series (96 rats)

Times (AO):	Donor	13 min
	Recipient	72 min
Weights (AO):	Donor	135 gm
	Recipient	267 gm
Mortality		26.6%
A. Technical Failure		
	- venous thrombosis	8.6%
	- arterial thrombosis	4.5%
	- bleeding	5.5%
B. Infection		8.0%

Table 2: Control Rat Grafts*

	Donor	Recipient	Survival (days)	Mean
1.	PVG($RT1^c$)	DA($RT1^a$)	6,7,7,6,8,7,7	6.8
2.	(PVGxDA)F_1	DA	9,9,10,8,8,6,10,9	8.6

* time of rejection taken as cessation of heart beat

Table 3: Unheated Cell Immunizations*

	Donor Heart	Recipient Immunized	Cell Type Immunization	Survival (days)	Mean Survival
1.	(PVGxDA)F_1	DA	(PVG T cells)	6,6,7,8,6,8,7,6,5	6.5[1]
2.	(PVGxDA)F_1	DA	(PVG B cells)	12,18,18,18,20,16, 11,17,20,16,26,19	17.6[3]
3.	(PVGxDA)F_1	DA	(PVGxDA)F_1 T cells	6,6,6,8,8,7,7,5,6	6.5[1]
4.	(PVGxDA)F_1	DA	(PVGxDA)F_1 B cells	9,12,14,11,18,17, 13,13,16,18	14.1[2]

1 Not significantly different from controls
2 $p < 0.05$
3 $p < 0.01$

Table 4: Heated Cell Immunizations (56°C)*

	Donor Heart	Recipient Immunized	Cell Type Immunization	Survival (days)	Mean Survival
1.	(PVGxDA)F_1	DA	PVG (T cells)	6,5,7,4,6,7,6	5.9[1]
2.	(PVGxDA)F_1	DA	PVG (B cells)	15,10,18,12,12,16	13.8[2]
3.	(PVGxDA)F_1	DA	F_1 (T cells)	6,6,5,5,7,8,7,7	6.4[1]
4.	(PVGxDA)F_1	DA	F_1 (B cells)	9,10,12,7,8,6,6, 12,13,15	9.8[1]

* 56°C heated for 30 minutes in water bath

1 Not significantly different from controls
2 $p < 0.05$
3 $p < 0.01$

Table 5: Heated Cell Immunizations (85°)*

	Donor Heart	Recipient Immunized	Cell Type Immunization	Survival (days)	Mean Survival
1.	(PVGxDA)F_1	DA	PVG (T cells)	4,6,8,8,9,9,8,7	7.4[1]
2.	(PVGxDA)F_1	DA	PVG (B cells)	10,16,10,12,30^+,30^+,30^+	19.7[2]
3.	(PVGxDA)F_1	DA	F_1 (T cells)	8,8,9,6,7,9,6,12	8.1[1]
4.	(PVGxDA)F_1	DA	F_1 (B cells)	10,16,19,18,19,12	15.7[2]

* heated 10 min 85°C water bath

1 Not significantly different from controls
2 $p < 0.05$
3 $p < 0.01$

Table 6: Mixed Cell Immunization

	Donor	Recipient	Cell Type	Survival	Mean
1.	(PVGxDA)F_1	DA	F_1 (T+B cells)	6,7,6,8	6.8
2.	(PVGxDA)F_1	DA	PVG (T+B cells)	7,8,7,7	7.3

Table 7: Platelet Immunization

	Donor	Recipient	Immunization	Survival	Mean
1.	(PVGxDA)F_1	DA	PVG (platelets)	8,9,10,10,11,9,8	9.2[1]
2.	(PVGxDA)F_1	DA	F_1 (platelets)	10,10,11,12,15,6, 10,8	10.3[1]
3.	(PVGxDA)F_1	DA	PVG (platelets 56°C)*	8,10,9,9	9.0[1]
4.	(PVGxDA)F_1	DA	F_1 (platelets 56°C)	10,8,8,9	8.8[1]

* 30 minutes

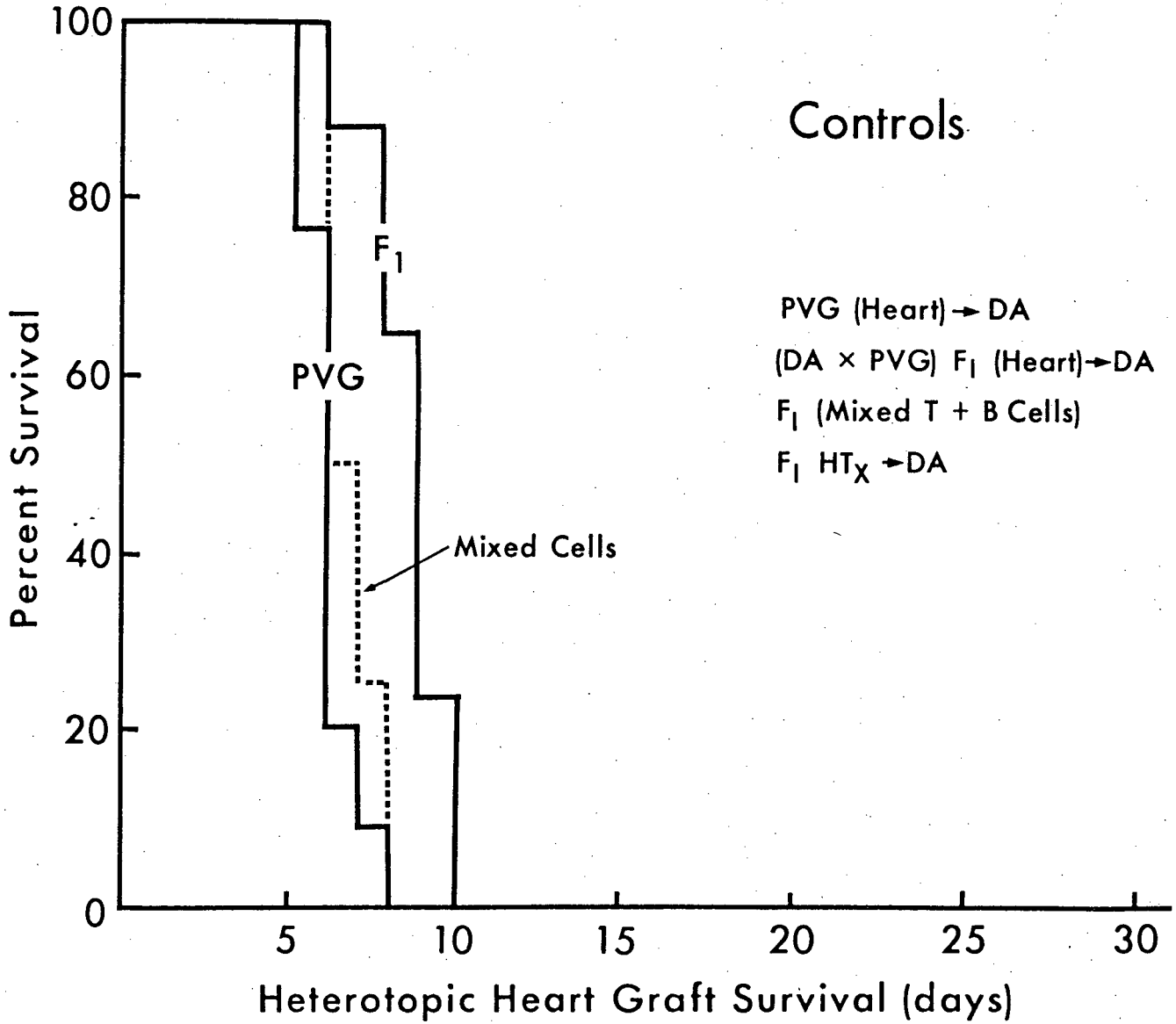
1 Not significantly different from controls

Table 8: Specificity Controls

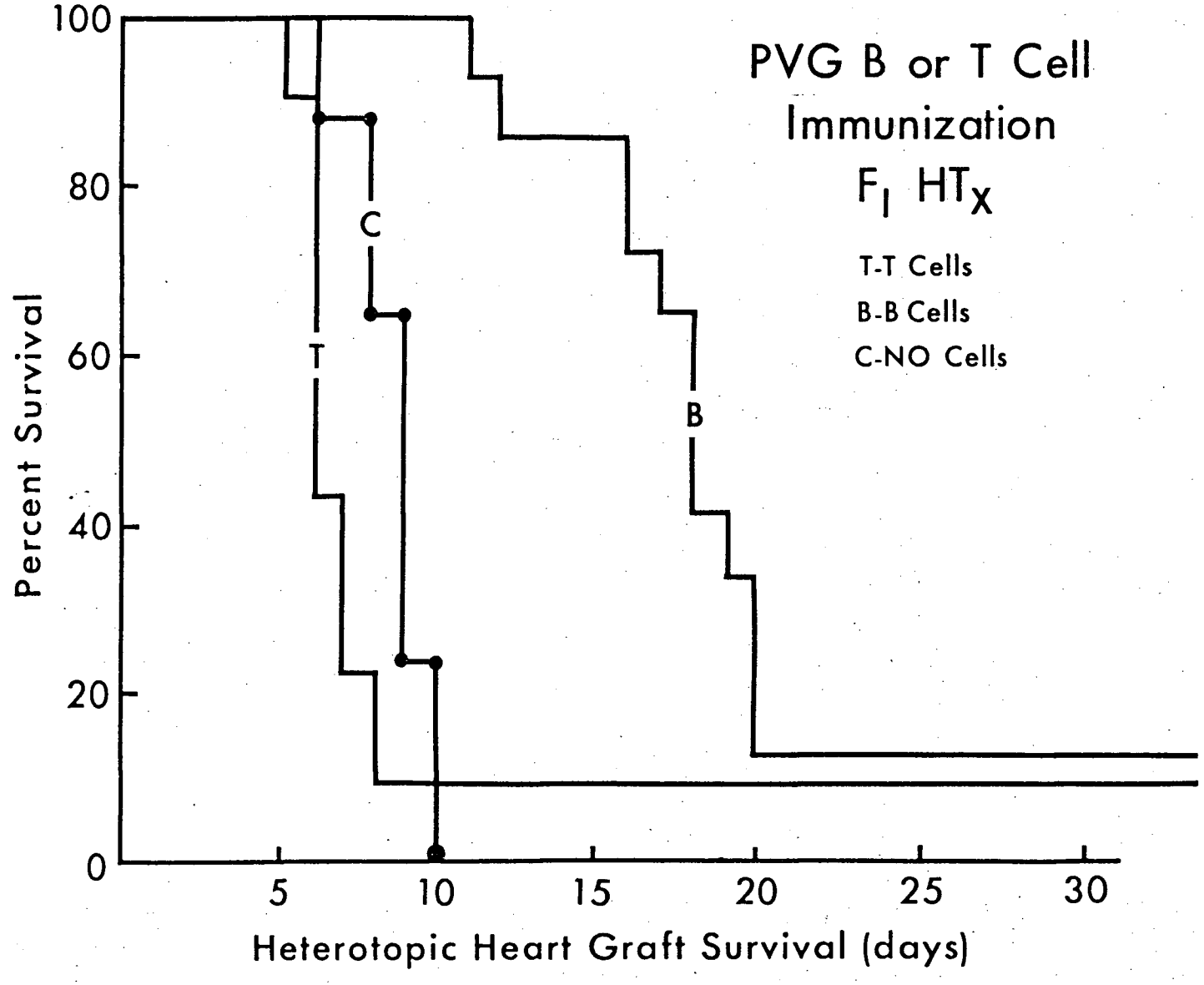
	Donor	Recipient	Survival (days)	Mean
1.	AO(RT1^{o})	DA	7,7,7,8	7.3
2.	AO	DA(IMM.F_1T)*	4,7,7,9,8	7.0
3.	AO	DA(IMM.F_1B)	7,7,8,8	7.5
4.	(PVGxDA)F_1	DA(IMM.AO_1T)	8,6,6,7	6.8
5.	(PVGxDA)F_1	DA(IMM.AOB)	4,8,8,7,7	6.8

* 10^7 cells injects 7 days prior to engraftment

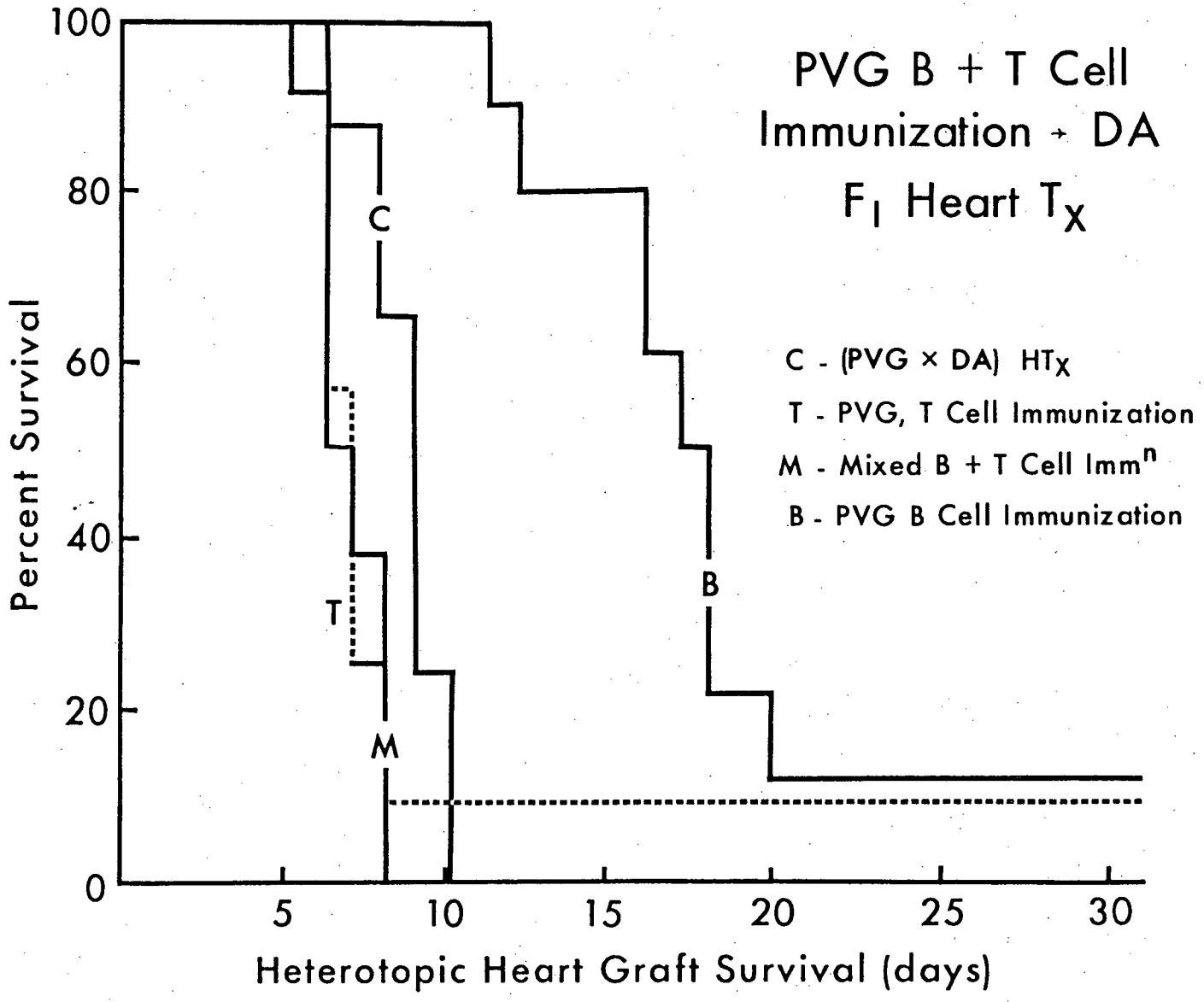
GRAPH 1


Controls
PVG (Heart) → DA
(DA × PVG) F1 (Heart) → DA
F1 (Mixed T + B Cells)
F1 HTX → DA
100
80
60
40
20
0
Percent Survival
F1
PVG
Mixed Cells
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)

GRAPH 2

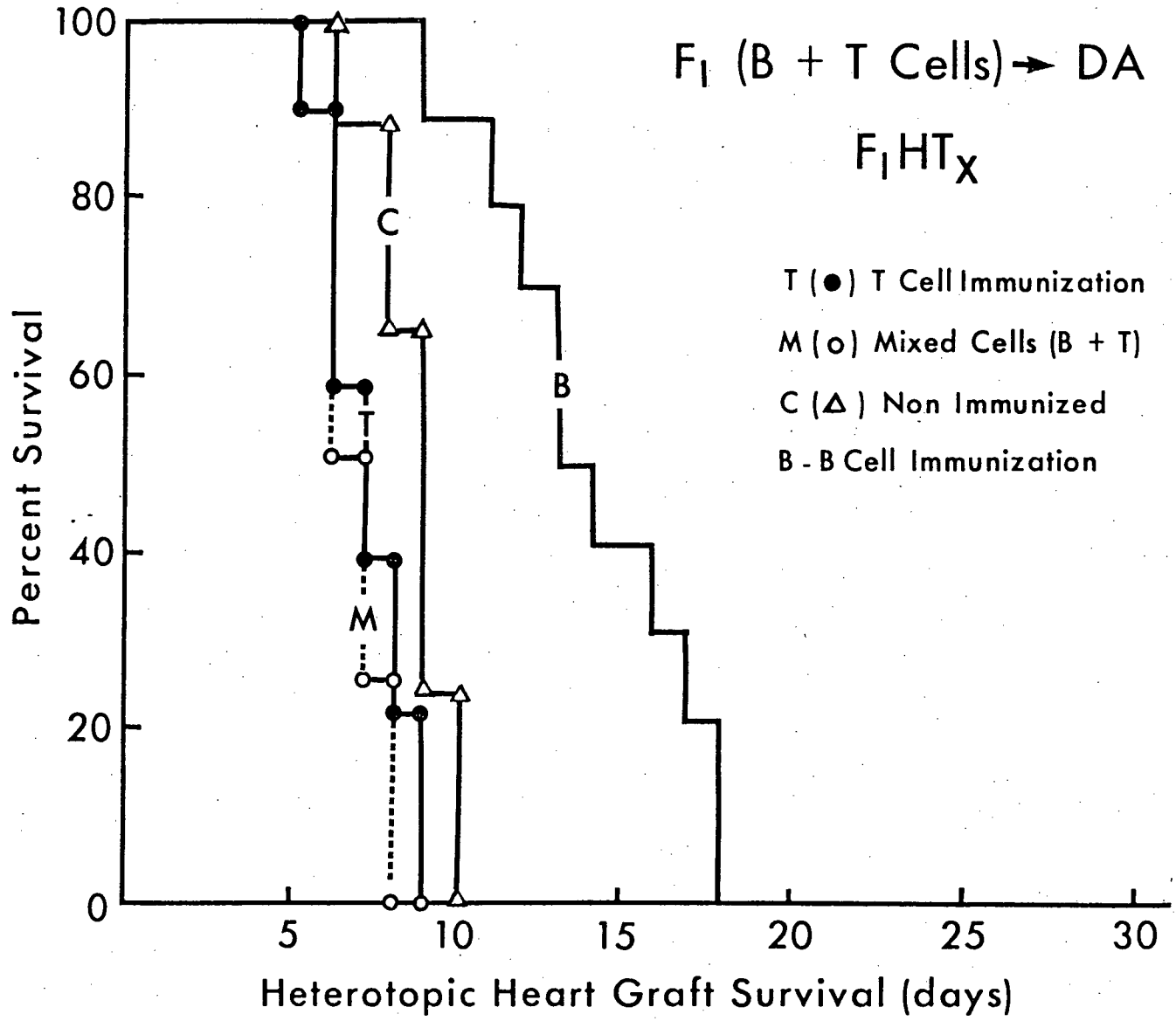

PVG B or T Cell
Immunization
F_1 HT_X
T-T Cells
B-B Cells
C-NO Cells
Percent Survival
100
80
60
40
20
0
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)
T
C
B

GRAPH 3

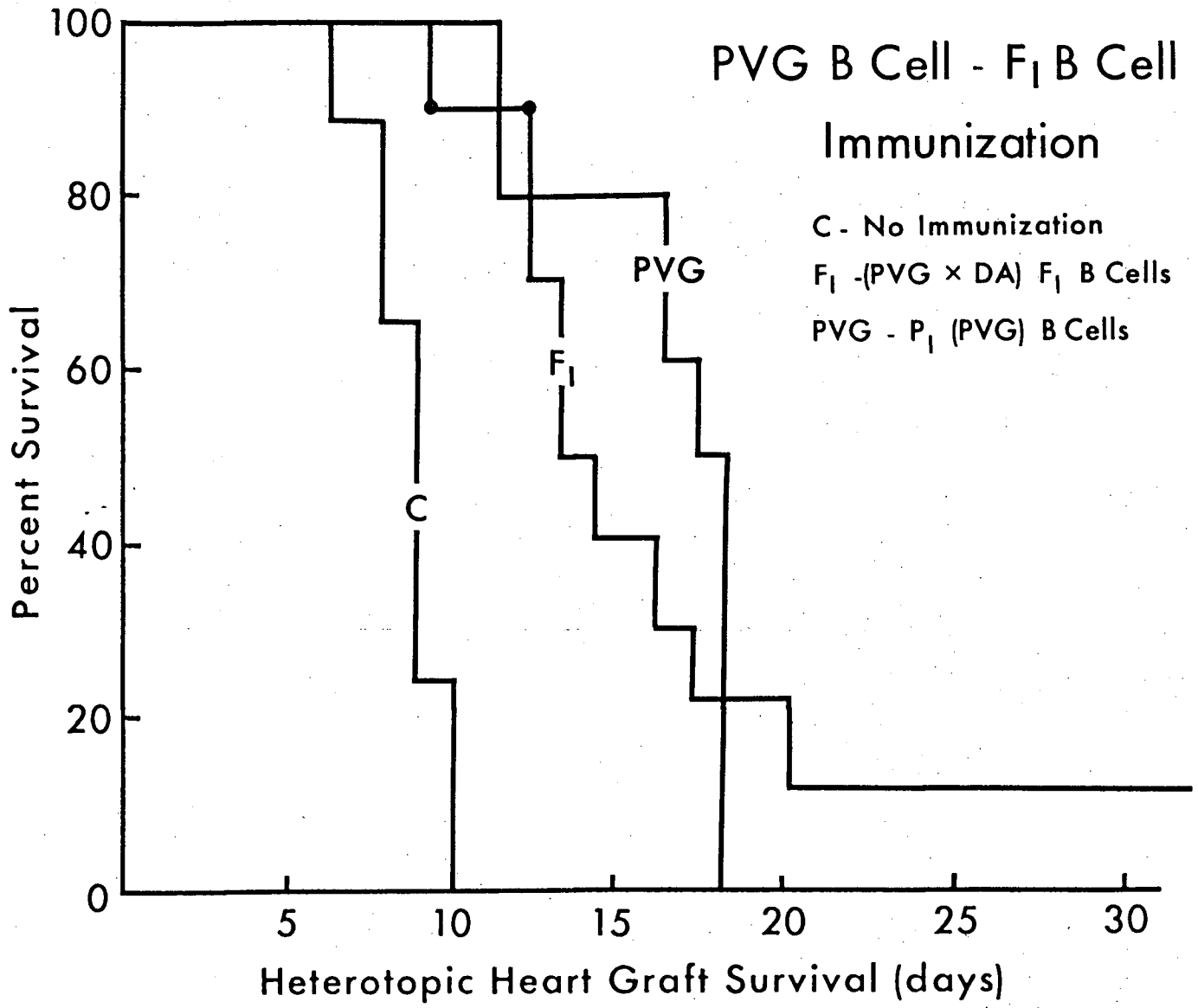

PVG B + T Cell
Immunization → DA
F1 Heart TX
C - (PVG × DA) HTX
T - PVG, T Cell Immunization
M - Mixed B + T Cell Immn
B - PVG B Cell Immunization
Percent Survival
100
80
60
40
20
0
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)
C
T
M
B

/69/

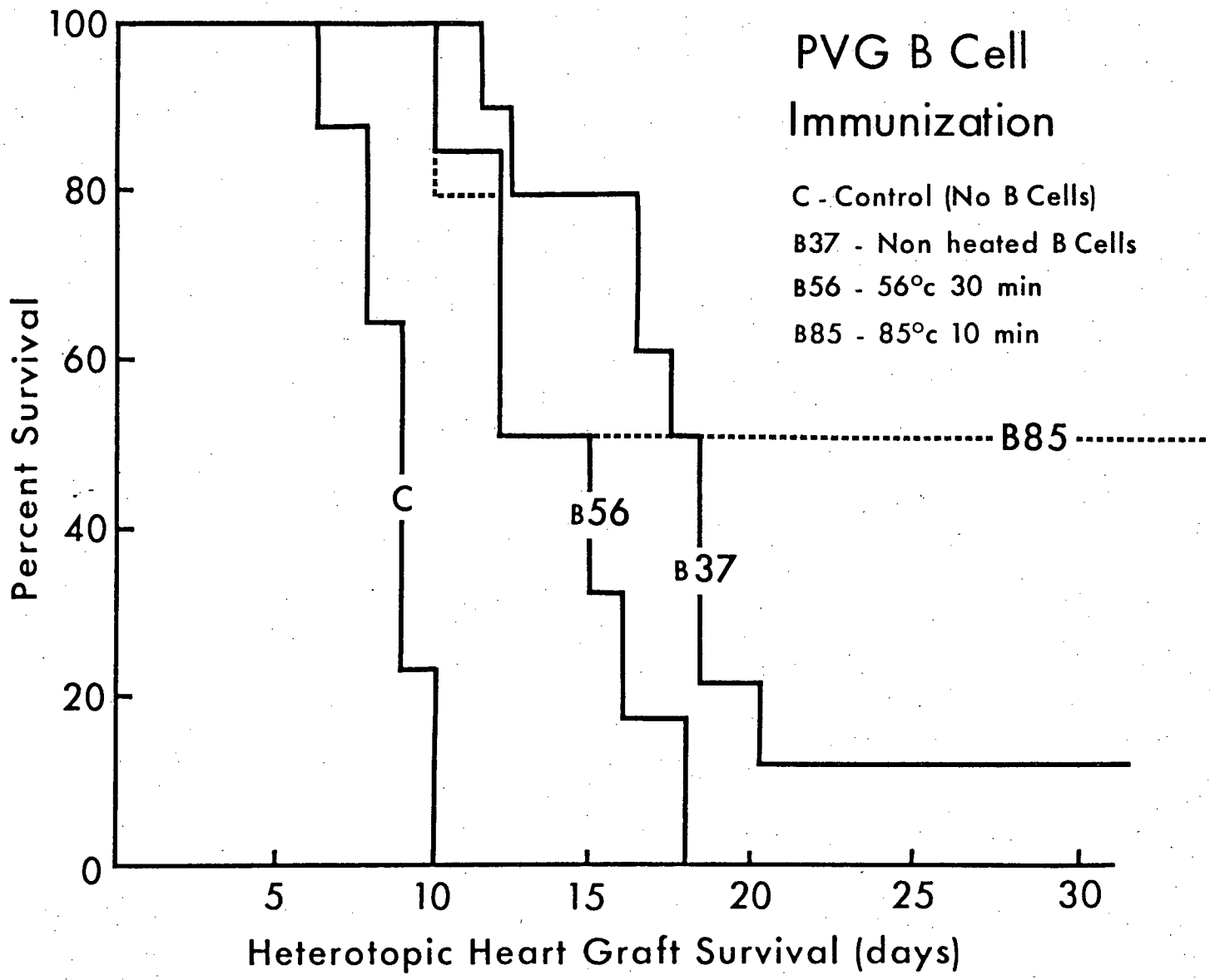
GRAPH 4


100
80
60
40
20
0
Percent Survival
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)
C
B
T
M
F1 (B + T Cells) → DA
F1HTx
T (●) T Cell Immunization
M (o) Mixed Cells (B + T)
C (△) Non Immunized
B - B Cell Immunization

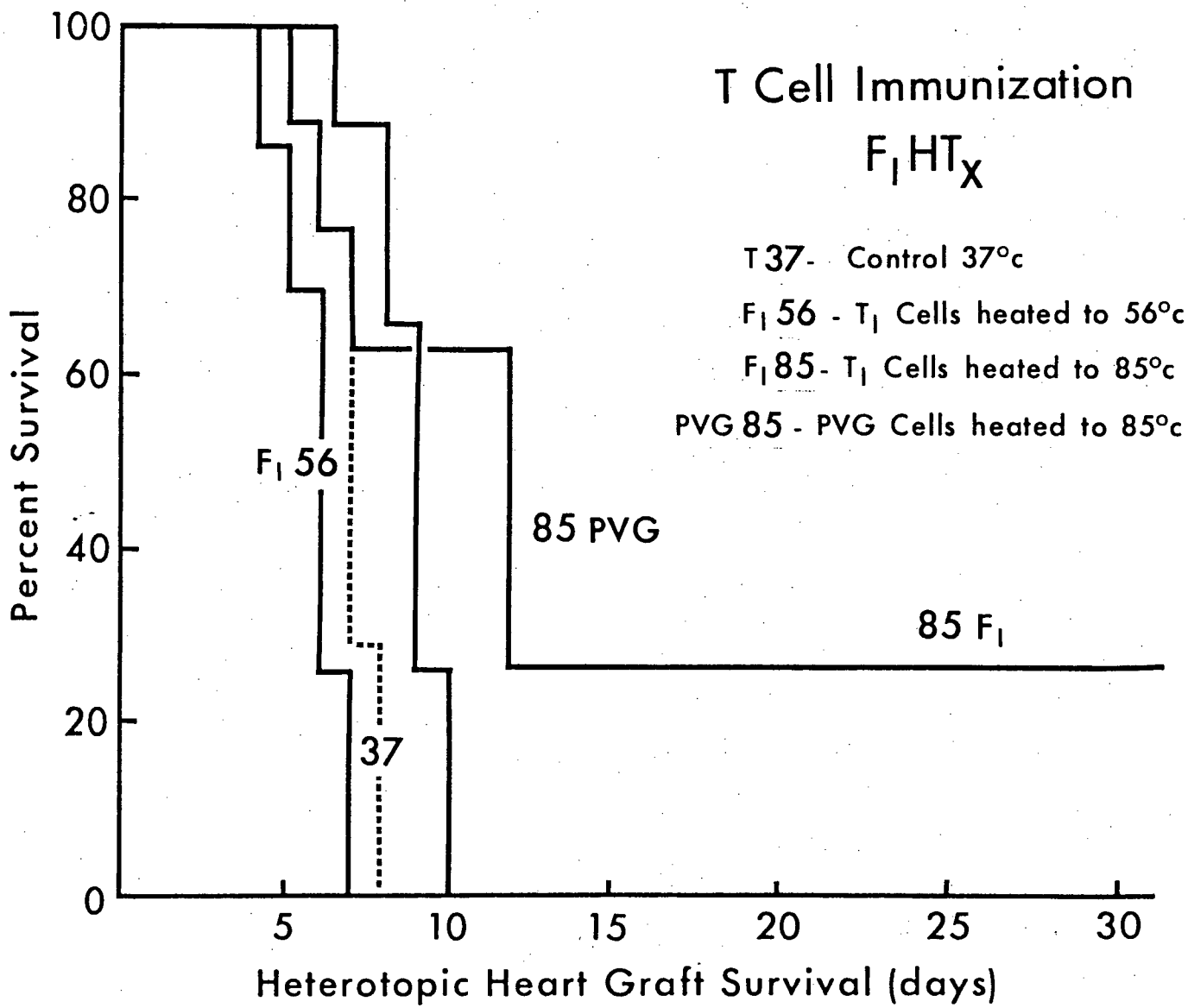
GRAPH 5


PVG B Cell - F1 B Cell
Immunization
C - No Immunization
F1 -(PVG × DA) F1 B Cells
PVG - P1 (PVG) B Cells
PVG
F1
C
Percent Survival
100
80
60
40
20
0
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)

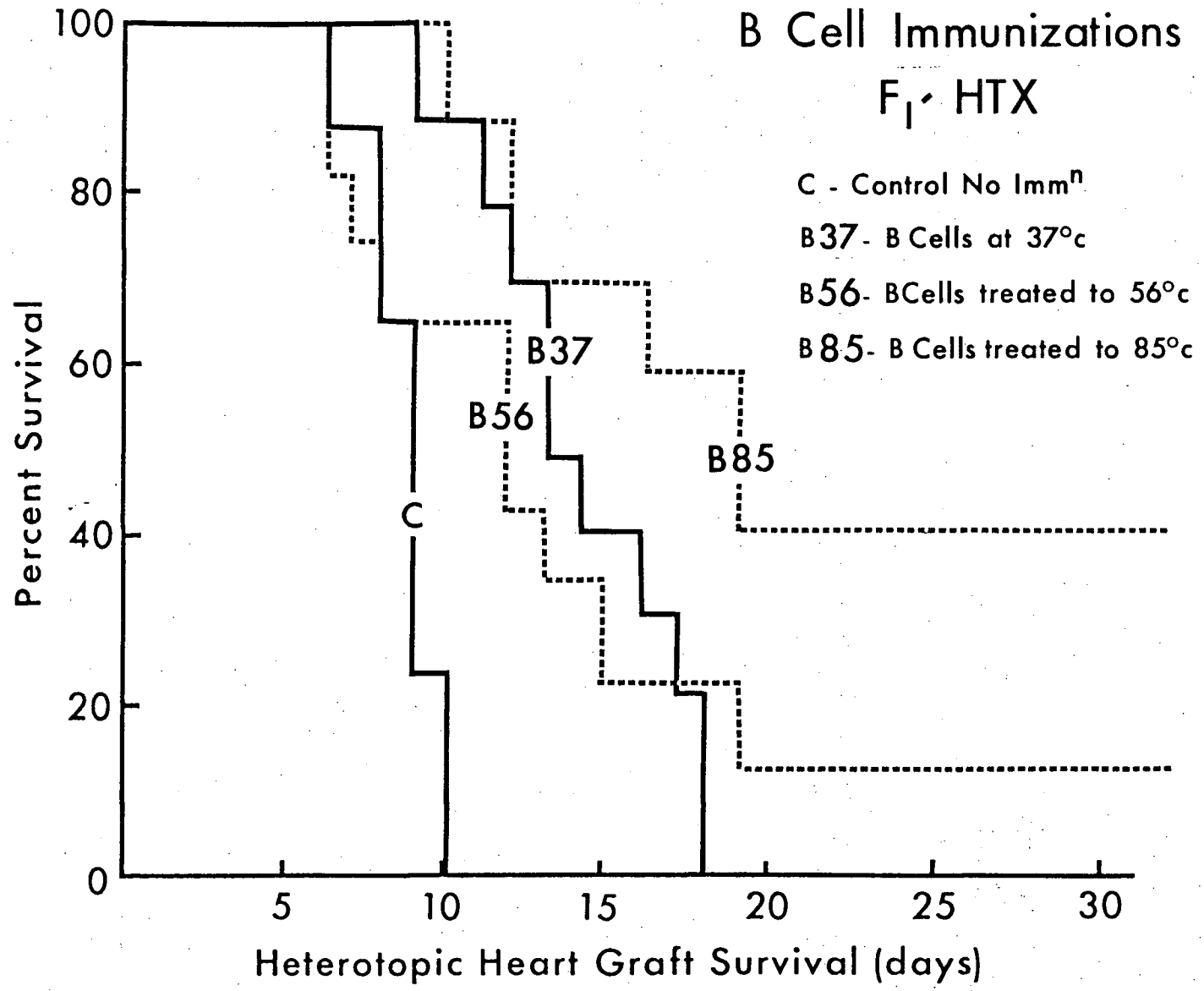
GRAPH 6


PVG B Cell
Immunization
C - Control (No B Cells)
B37 - Non heated B Cells
B56 - 56°c 30 min
B85 - 85°c 10 min
Percent Survival
100
80
60
40
20
0
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)
C
B56
B37
B85

GRAPH 7


T Cell Immunization
F_1HT_X
T 37 - Control 37°c
F_1 56 - T_1 Cells heated to 56°c
F_1 85 - T_1 Cells heated to 85°c
PVG 85 - PVG Cells heated to 85°c
Percent Survival
100
80
60
40
20
0
F_1 56
85 PVG
85 F_1
37
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)

GRAPH 8


B Cell Immunizations
F_1 - HTX
C - Control No Imm^n
B37 - B Cells at 37°c
B56 - BCells treated to 56°c
B85 - B Cells treated to 85°c
B37
B56
B85
C
Percent Survival
100
80
60
40
20
0
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)

GRAPH 9

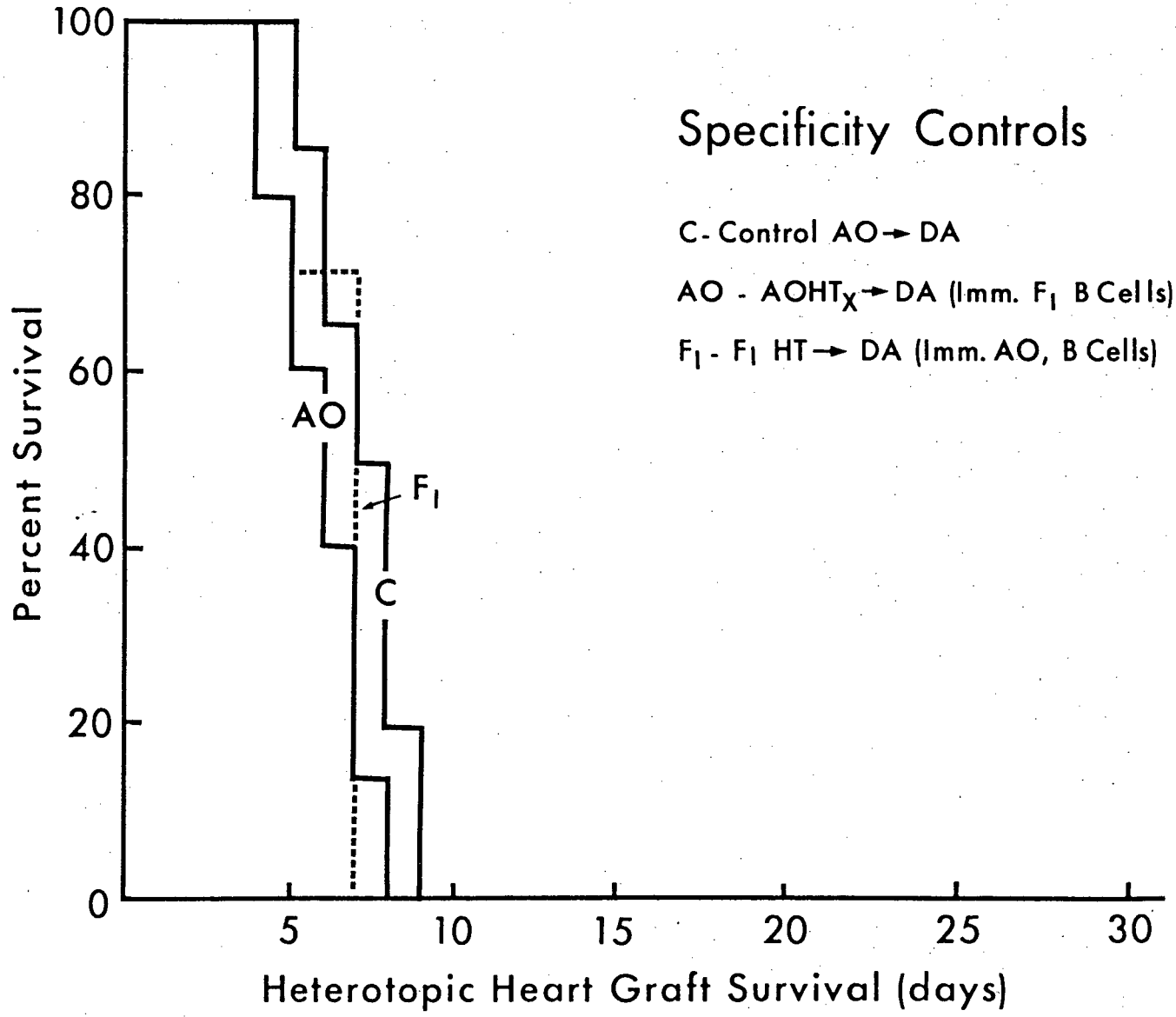

Specificity Controls
C- Control AO → DA
AO - AOHTx → DA (Imm. F1 B Cells)
F1 - F1 HT → DA (Imm. AO, B Cells)
Percent Survival
100
80
60
40
20
0
AO
F1
C
5
10
15
20
25
30
Heterotopic Heart Graft Survival (days)

FIGURE 1
THE CUFF VASCULAR ANASTOMOSIS SET SIDE BY SIDE

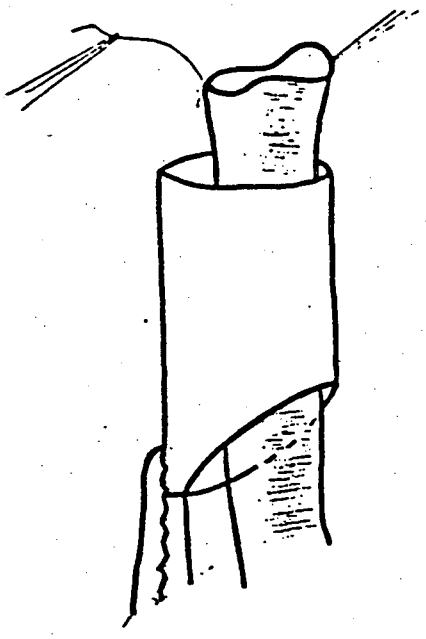


FIG 1A - THROUGH THE POLYETHYLENE CUFF, THE RECIPIENT VESSEL IS PULLED.

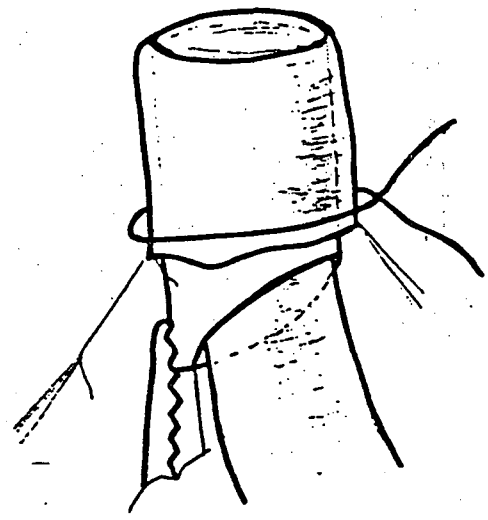


FIG 1B - THE RECIPIENT VESSEL IS FOLDED OVER THE CUFF AND LIGATED IN PLACE.

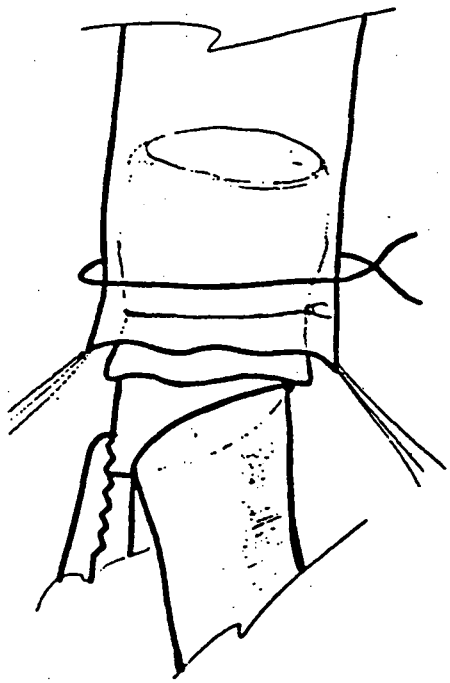


FIG 1C - THE CUFF IS INVAGINATED INTO THE DONOR VESSEL AND LIGATED. NOTE THAT ONLY INTIMA IS IN CONTACT WITH BLOOD FLOW.

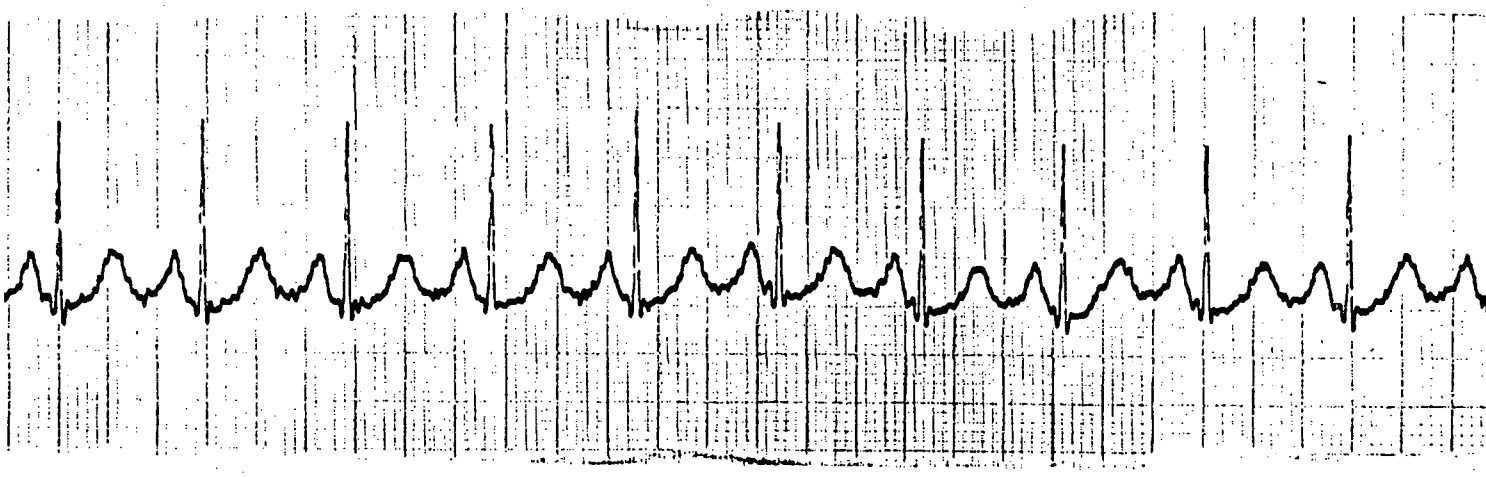


FIG 2 - ECG OF HEART AFTER GRAFTING.

CHART SPEED 2X NORMAL.

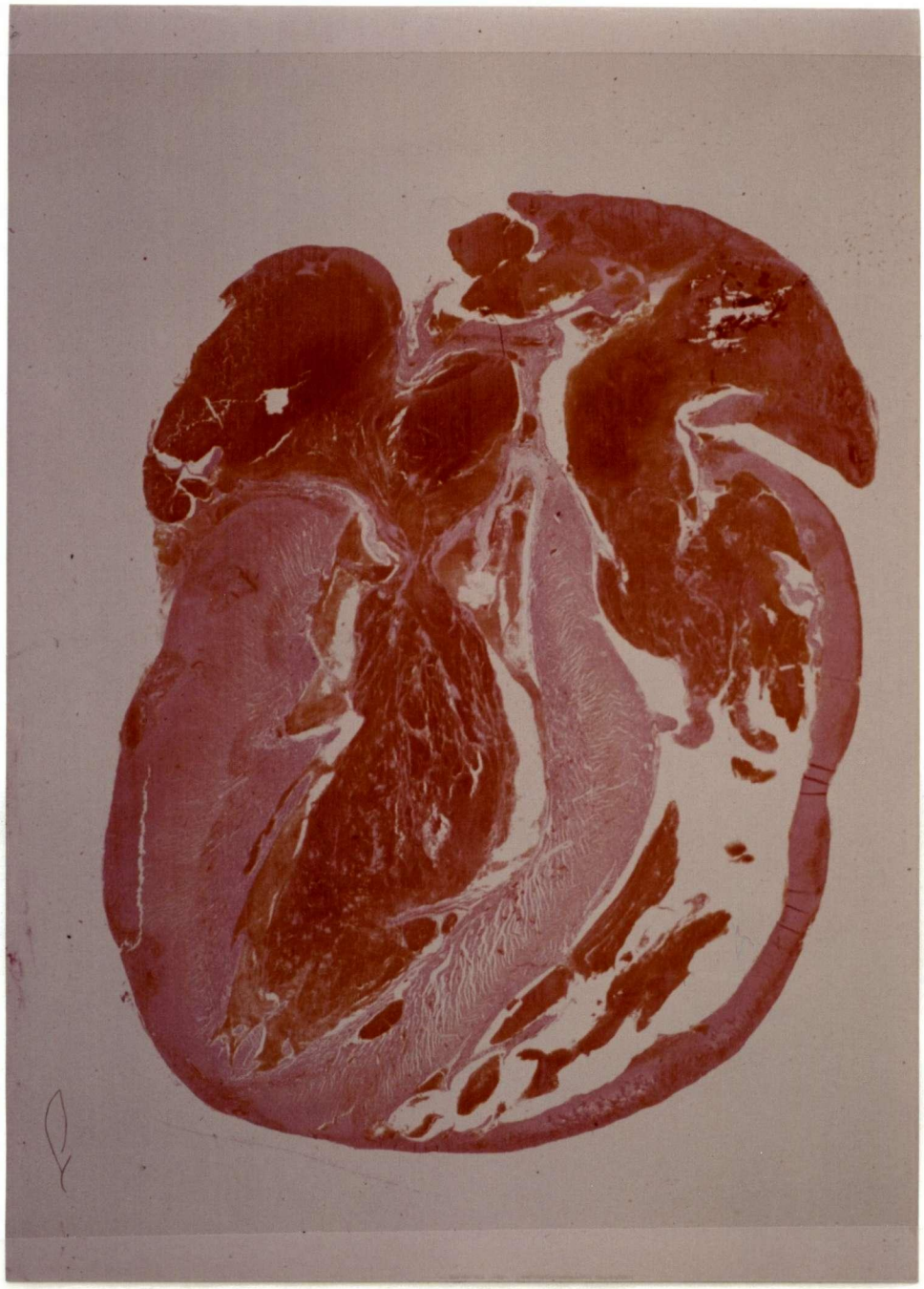


FIG 3 - REMOVED RAT HEART TRANSPLANT AFTER VENOUS INFARCTION POST-OP DAY 2, SHOWING ACUTE VENOUS CLOT.

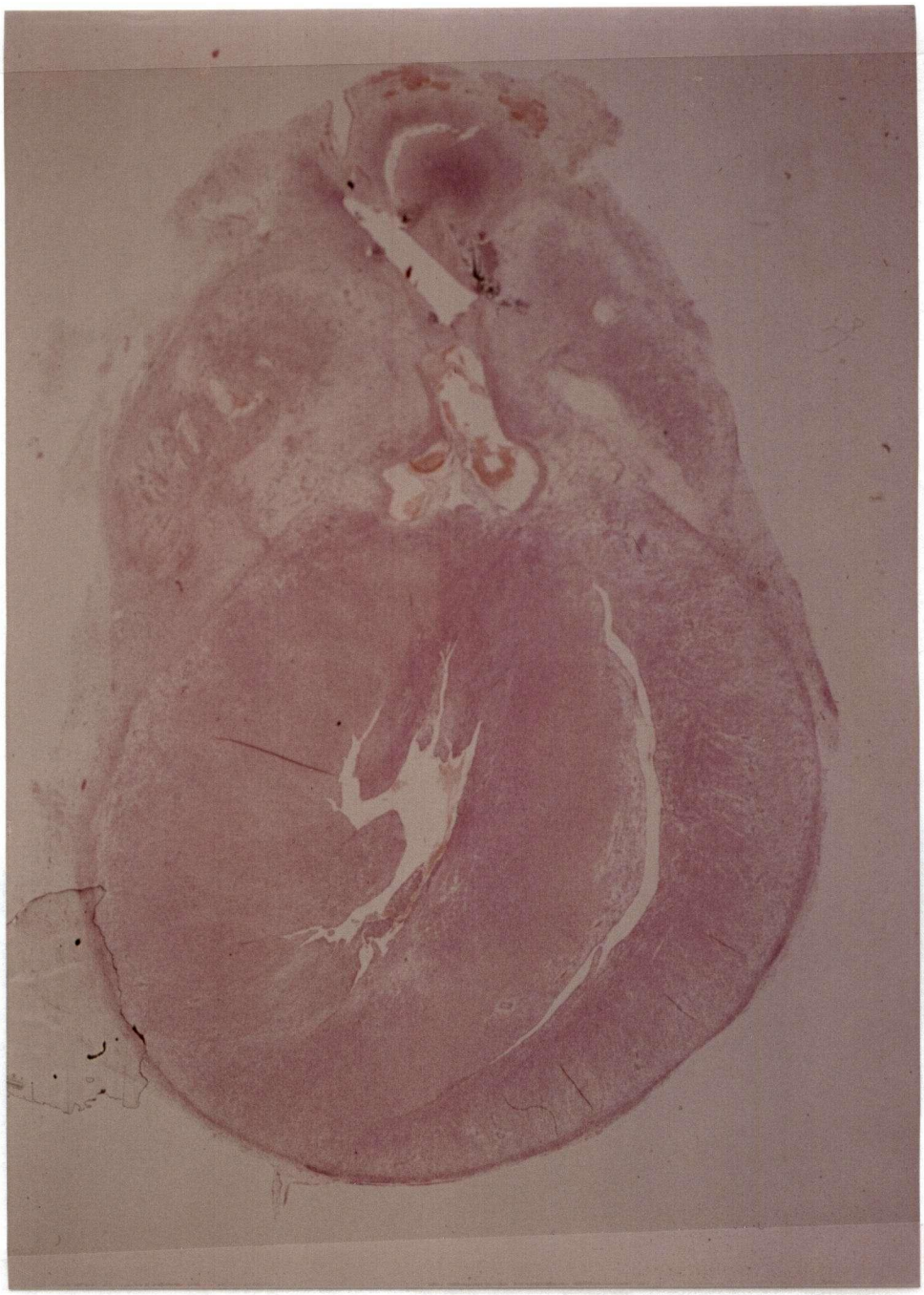


FIG 4 - REMOVED RAT HEART TRANSPLANT SHOWING ACUTE ARTERIAL CLOT DAY 1.

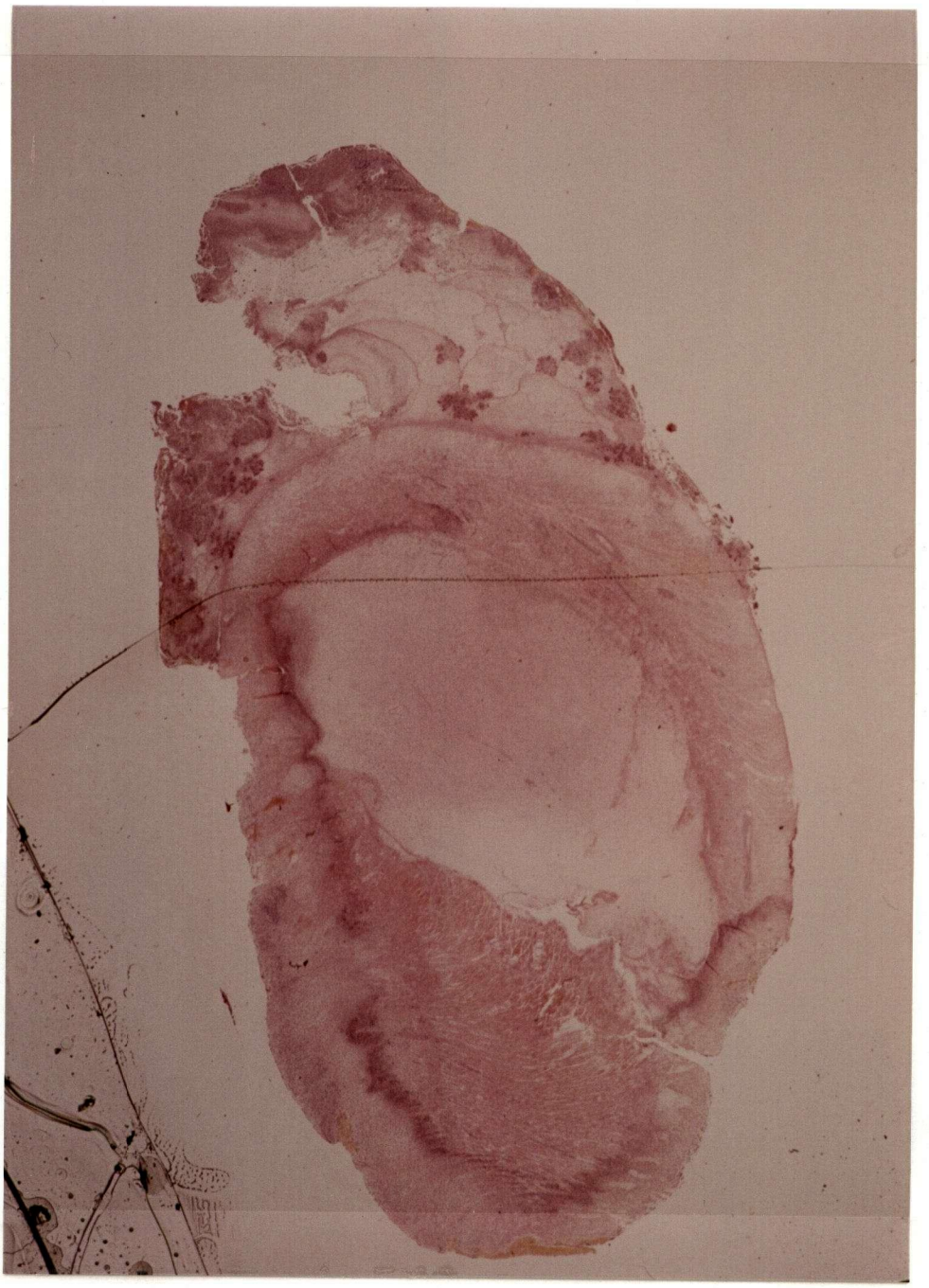


FIG 5 - REMOVED RAT HEART TRANSPLANT DAY 6. SOME REJECTION CHANGES ARE EVIDENT, BUT THE ANIMAL DIED OF PNEUMONIA. GRAFT FUNCTIONING AT TIME OF DEATH.

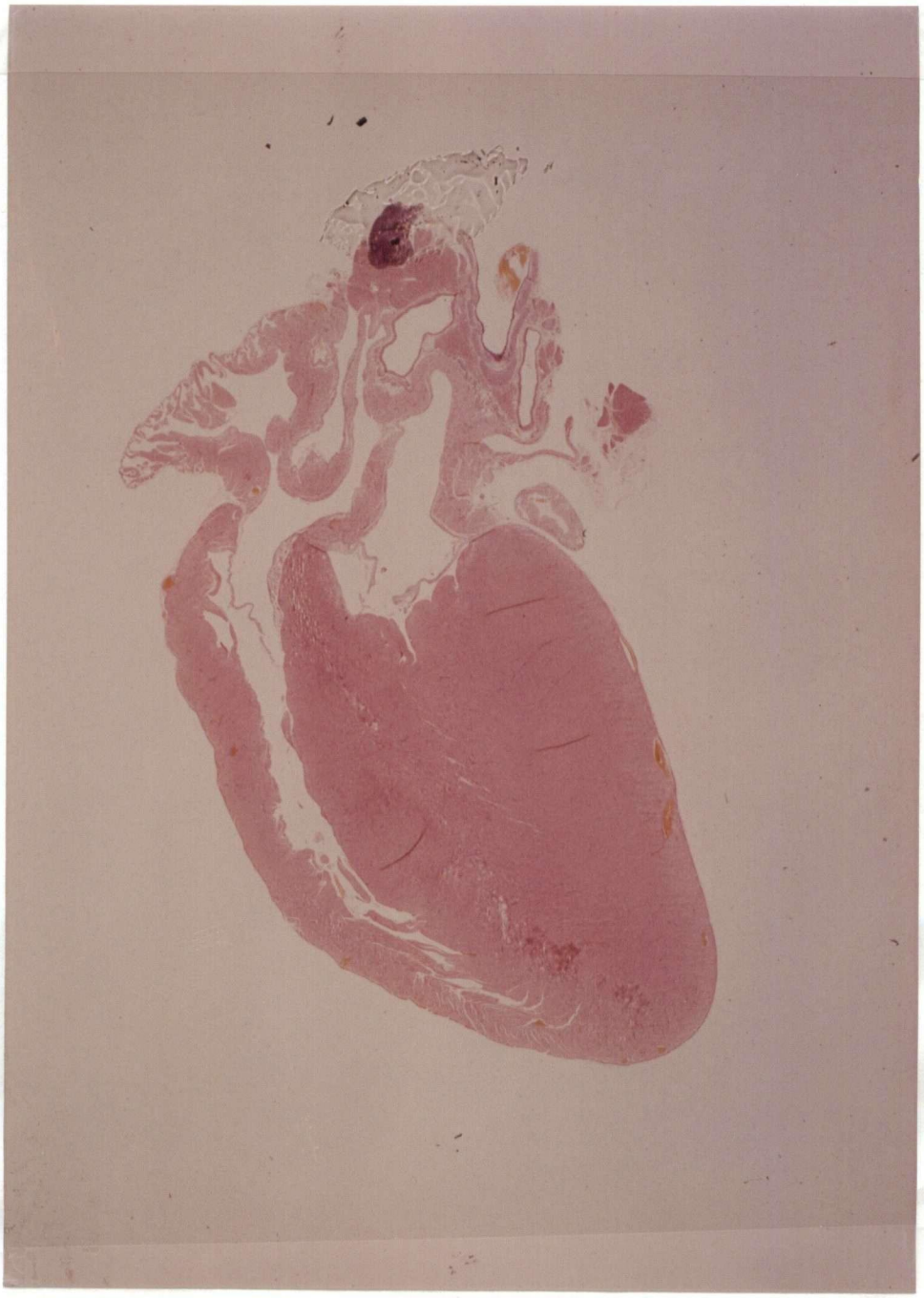


FIG 6 - NORMAL HEART, NOT TRANSPLANTED.

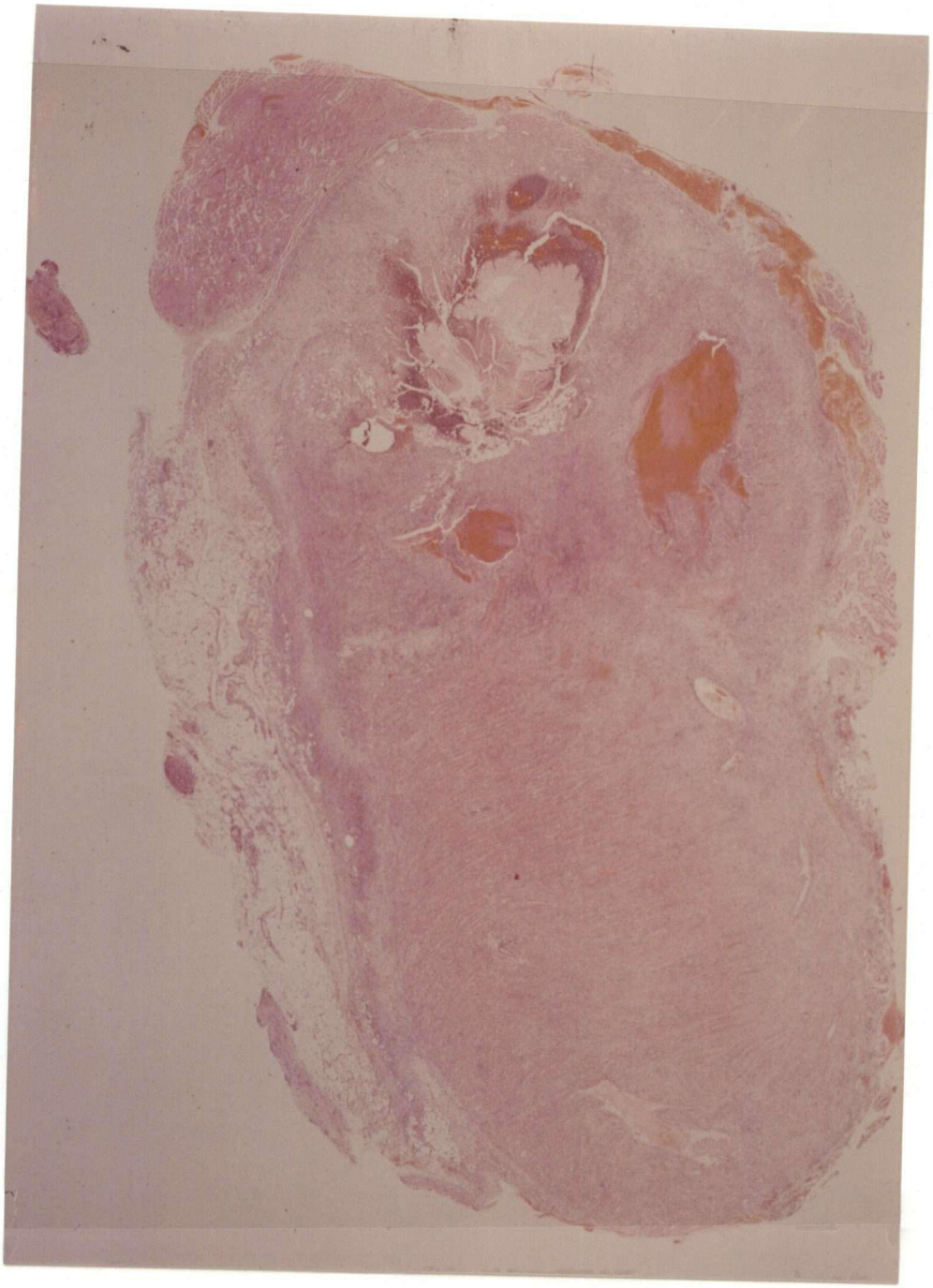


FIG 7 - REMOVED RAT HEART TRANSPLANT AFTER 14 DAYS. ACUTE ALLOGRAFT REJECTION.

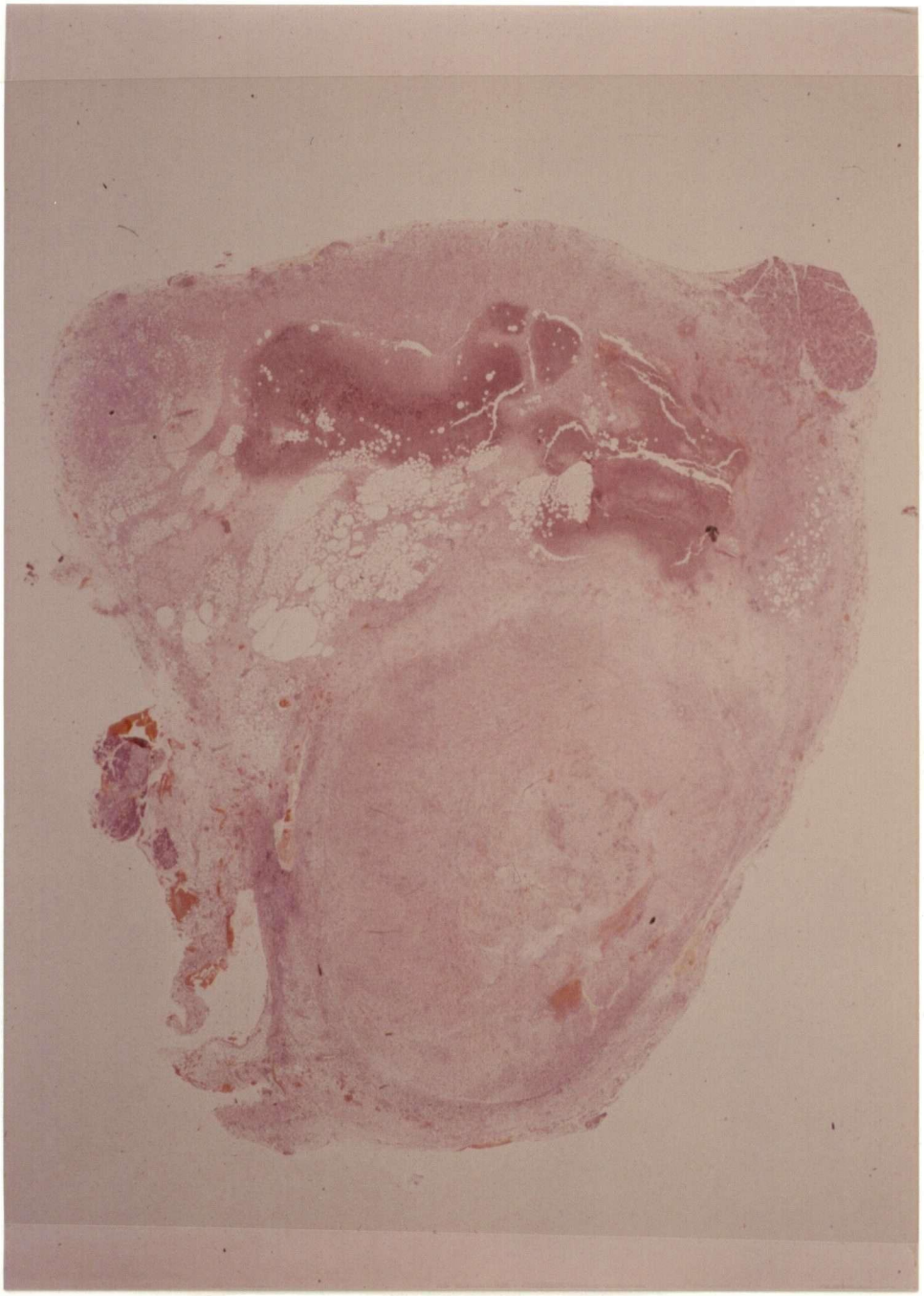
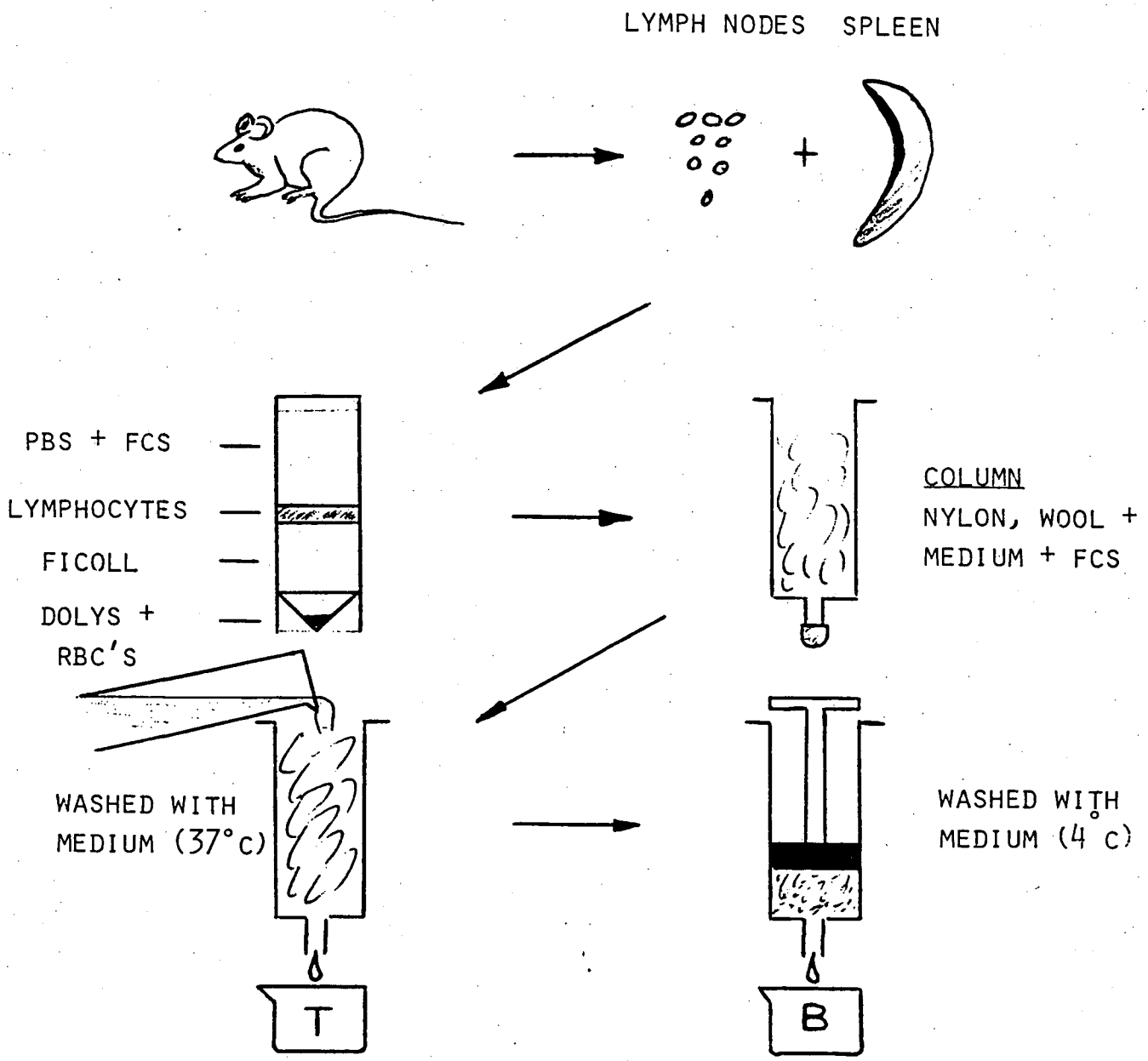


FIG 8 - RAT HEART ALLOGRAFT REJECTED 8 DAYS, REMOVED 60 DAYS.

FIGURE 9

CELL SEPARATION TECHNIQUE

FICOLL-ISOPAQUE/NYLON WOOL



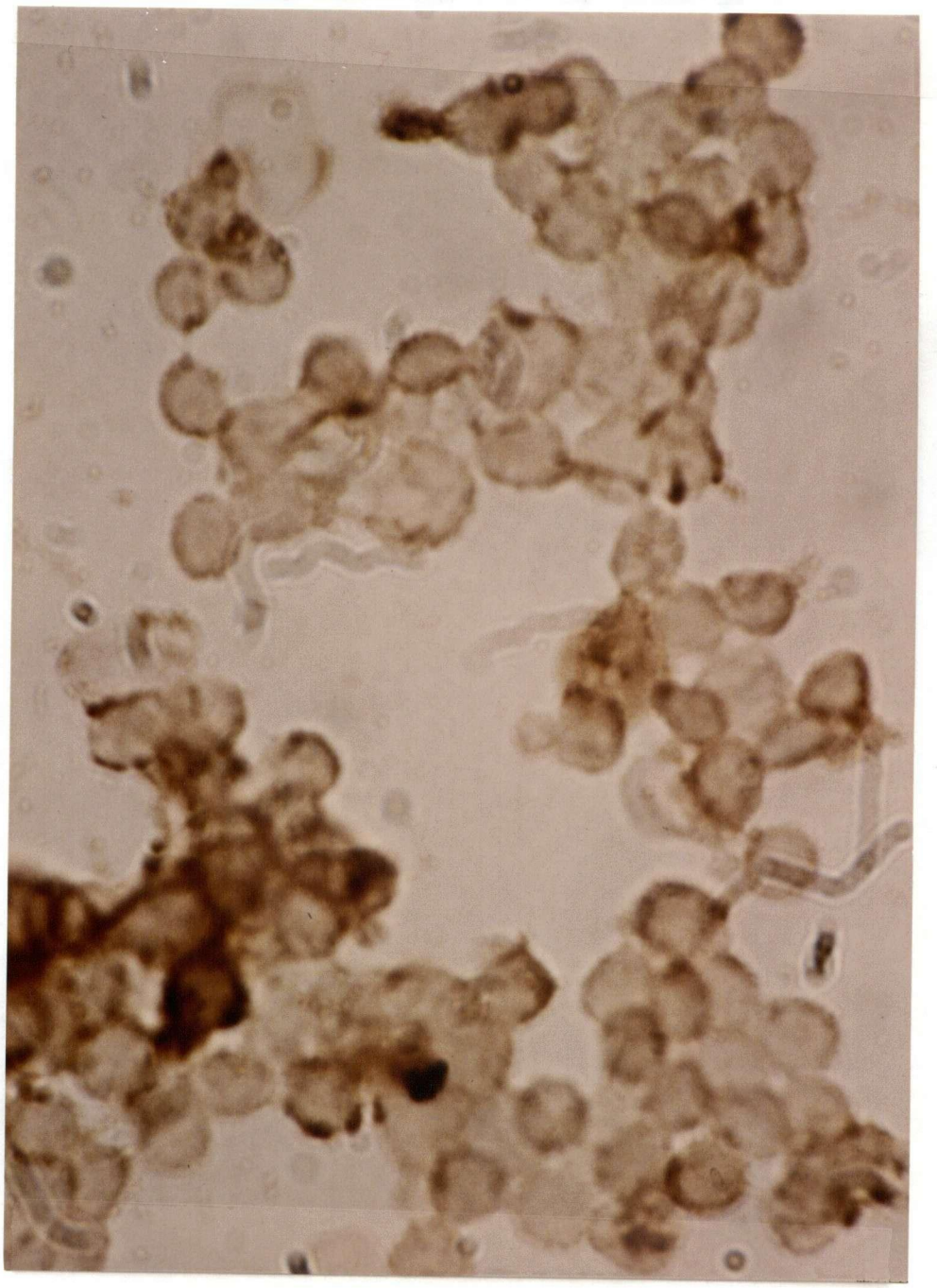


FIG 10A - PEROXIDASE STAIN (T CELLS)

T CELLS - FEW CELLS WITH DARK HALO. T CELLS DO NOT STAIN. VERY DARK CELLS ARE DEAD.

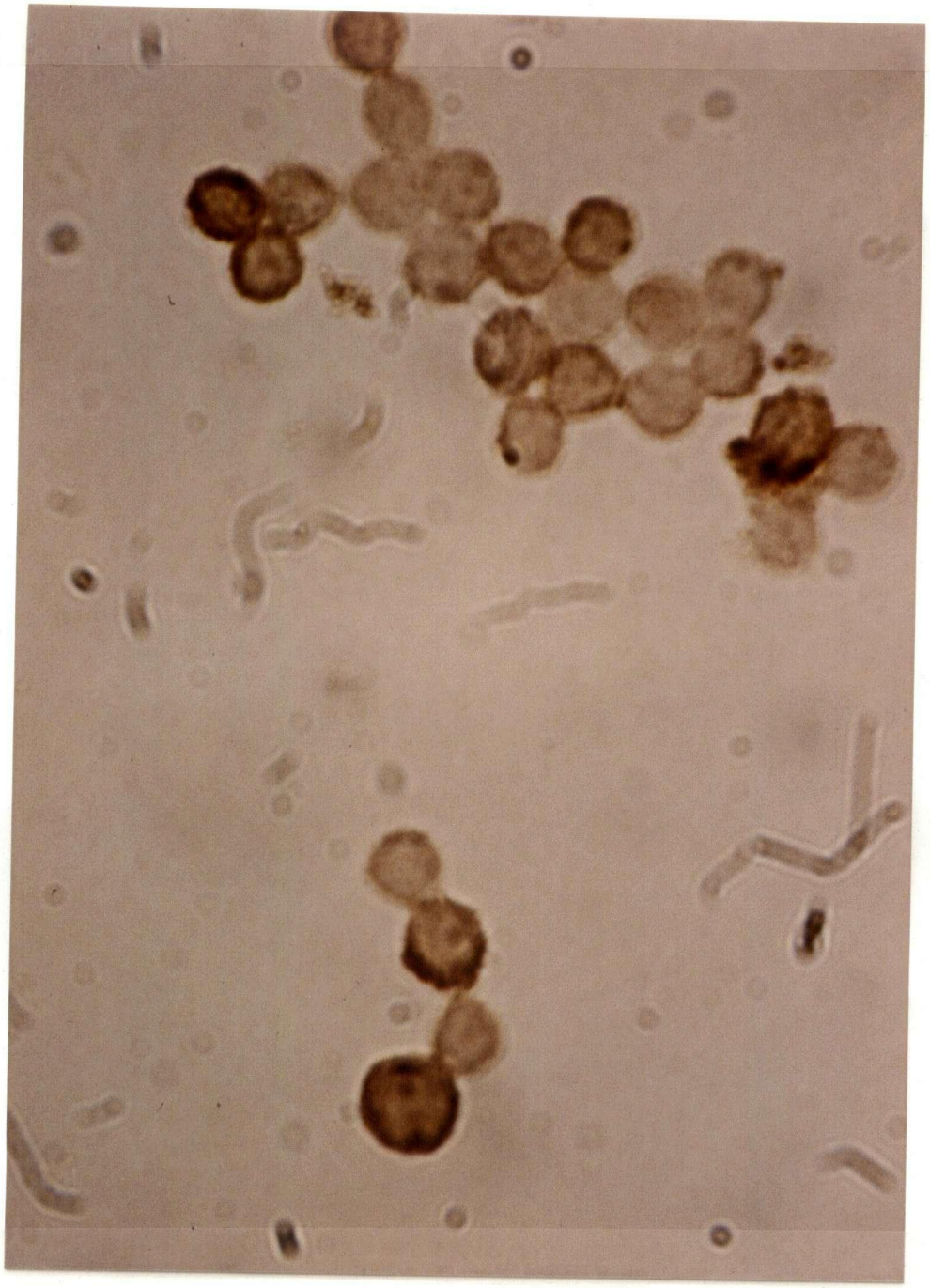


FIG 10B - PEROXIDASE STAIN (B CELLS)

B CELLS - MOST HAVE TAKEN UP STAIN.

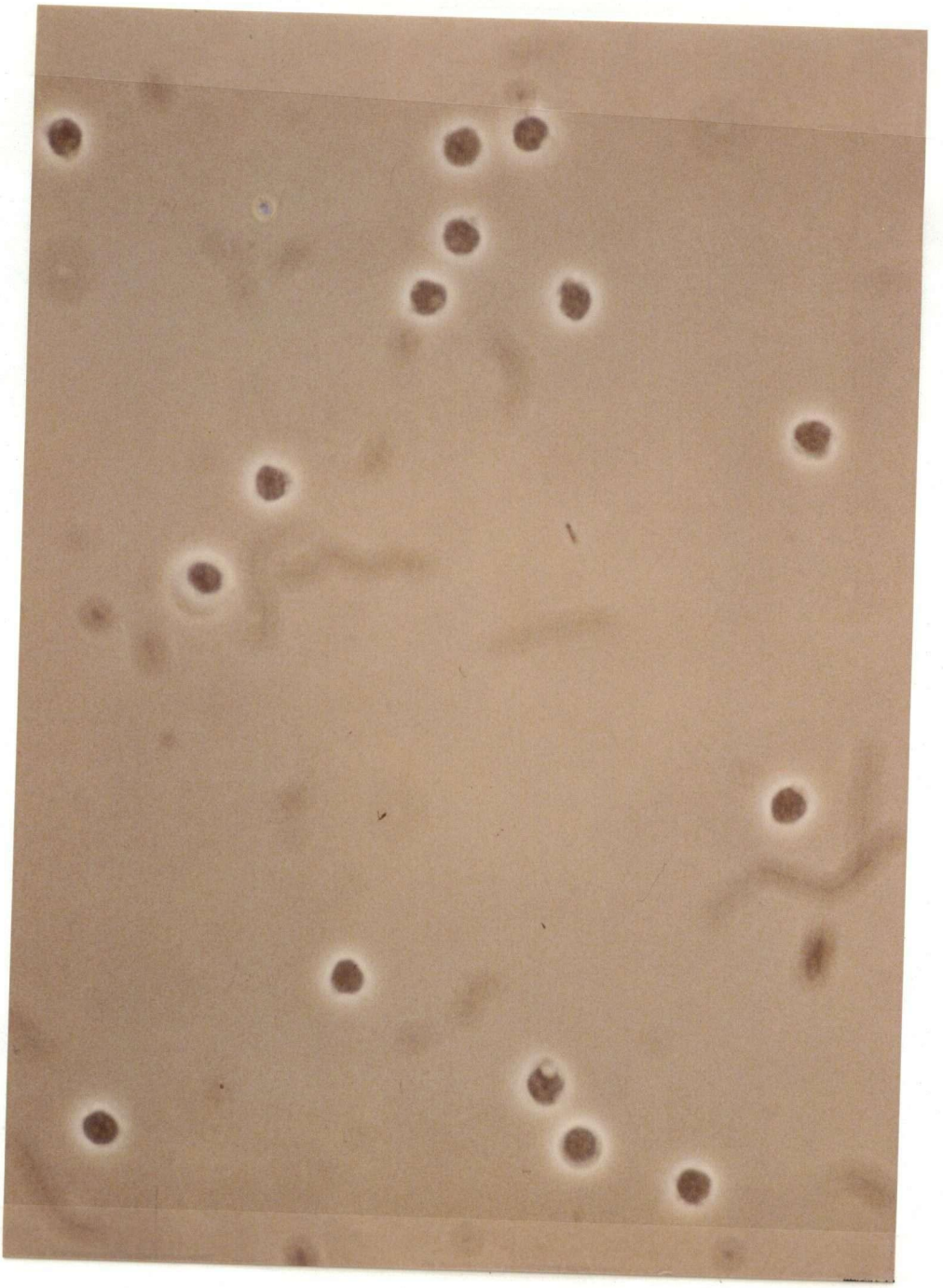


FIG 11A - B CELLS, PHASE CONTRAST

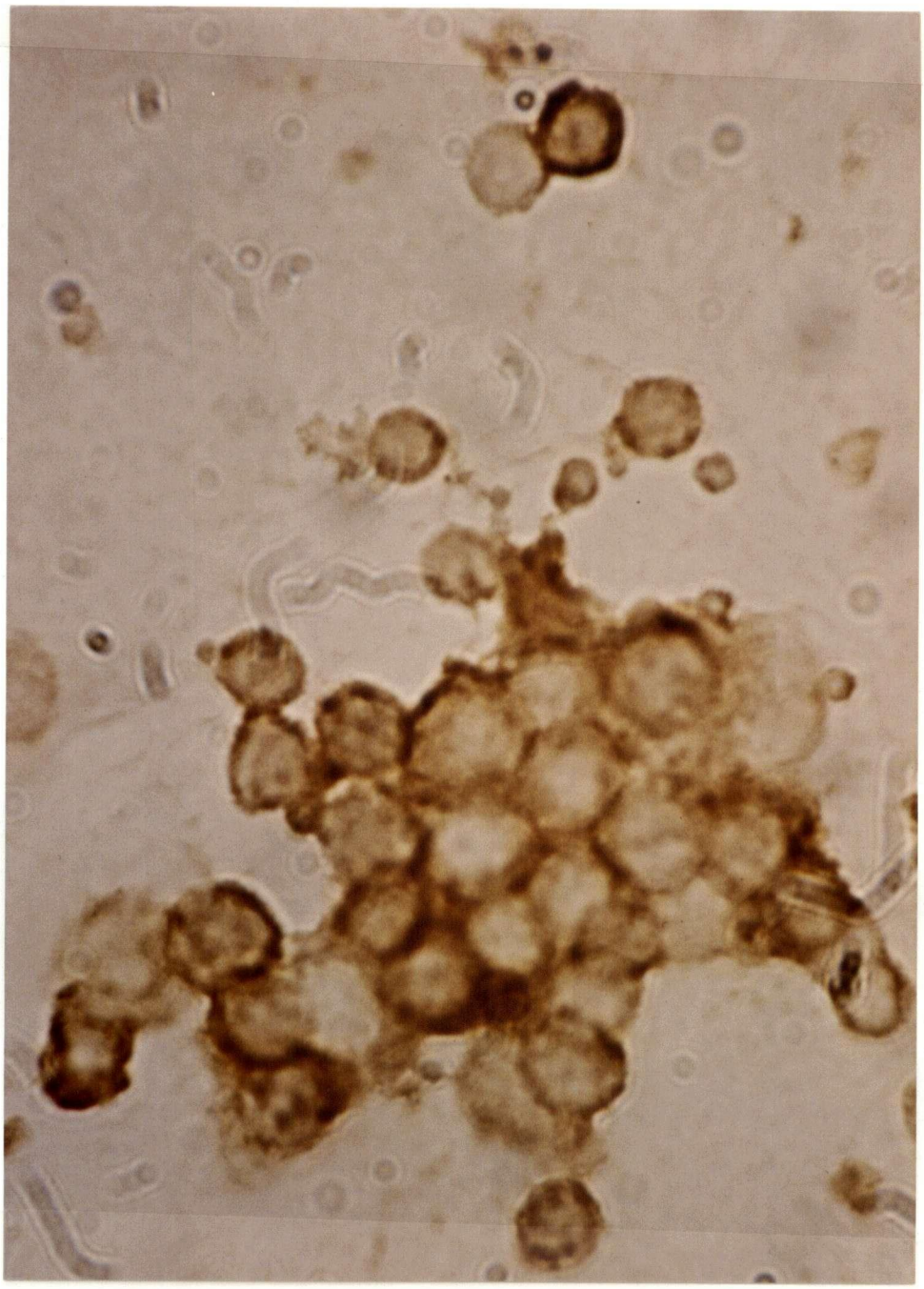


FIG 11B - PEROXIDASE STAIN (B CELLS)

TO DEMONSTRATE LACK OF CONTAMINATION.

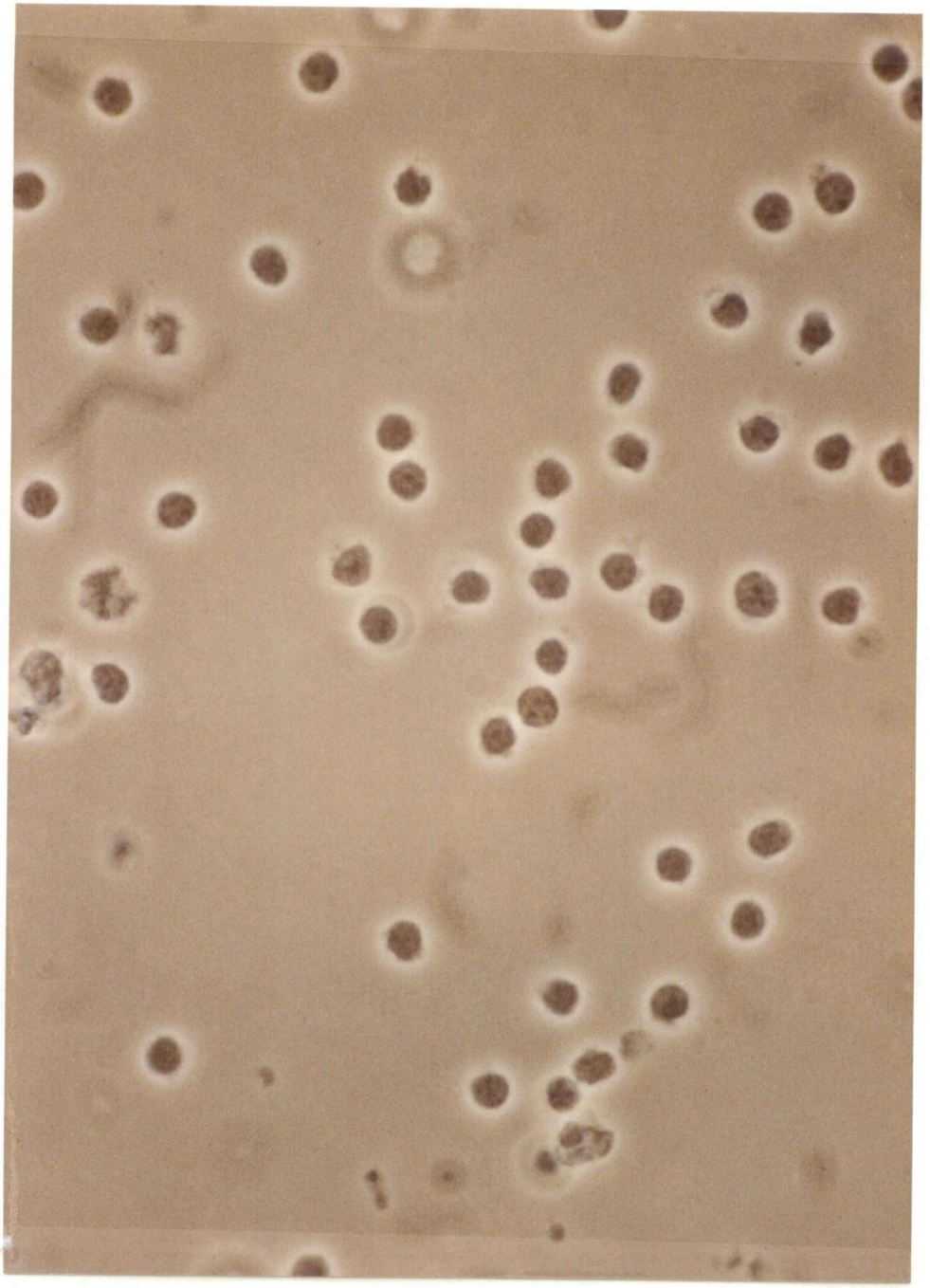


FIG 12A - PHASE CONTRAST OF T CELLS

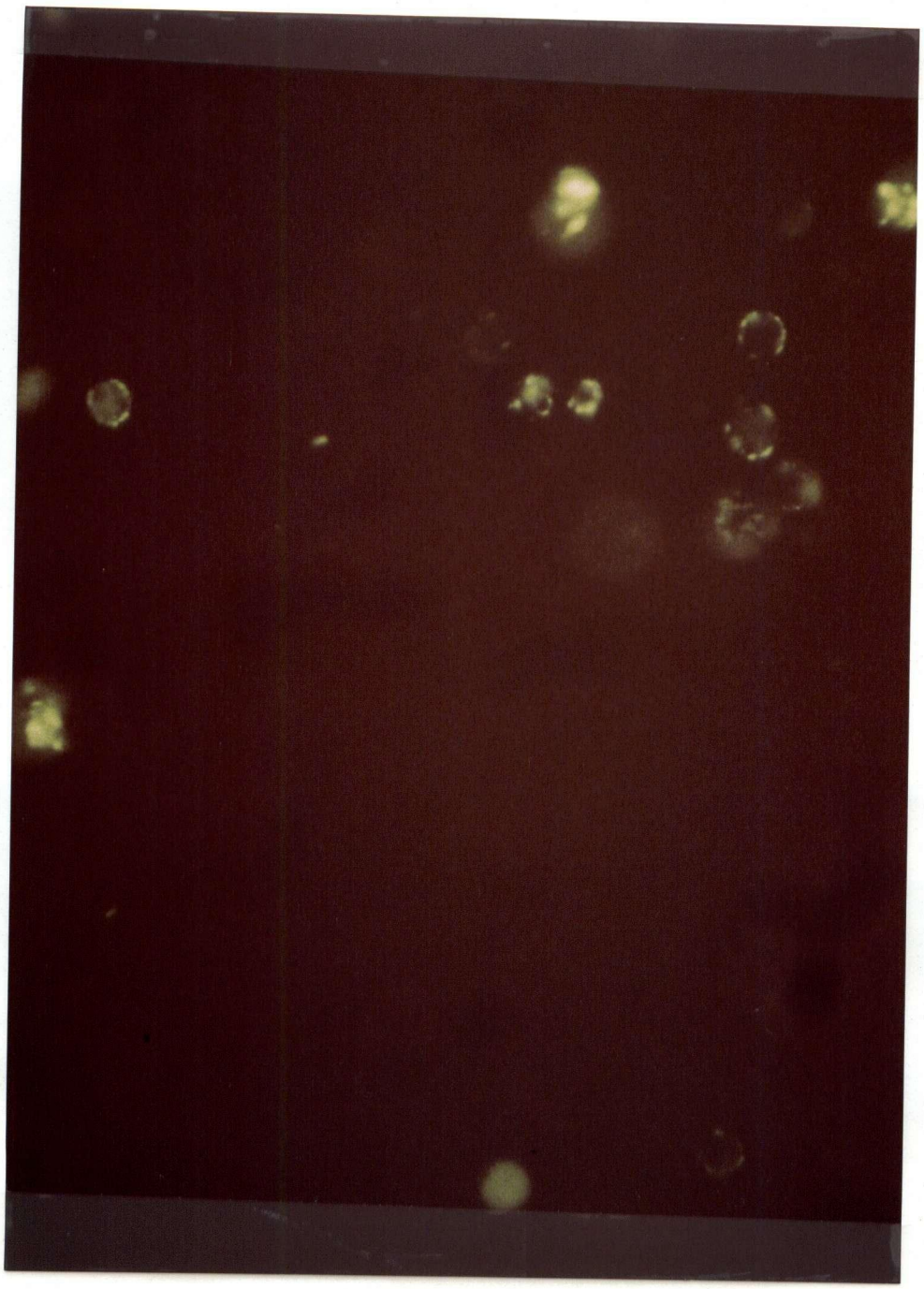


FIGURE 12B - FLUORESCEIN STAIN (T CELLS)

IMAGES ARE DEAD CELLS.

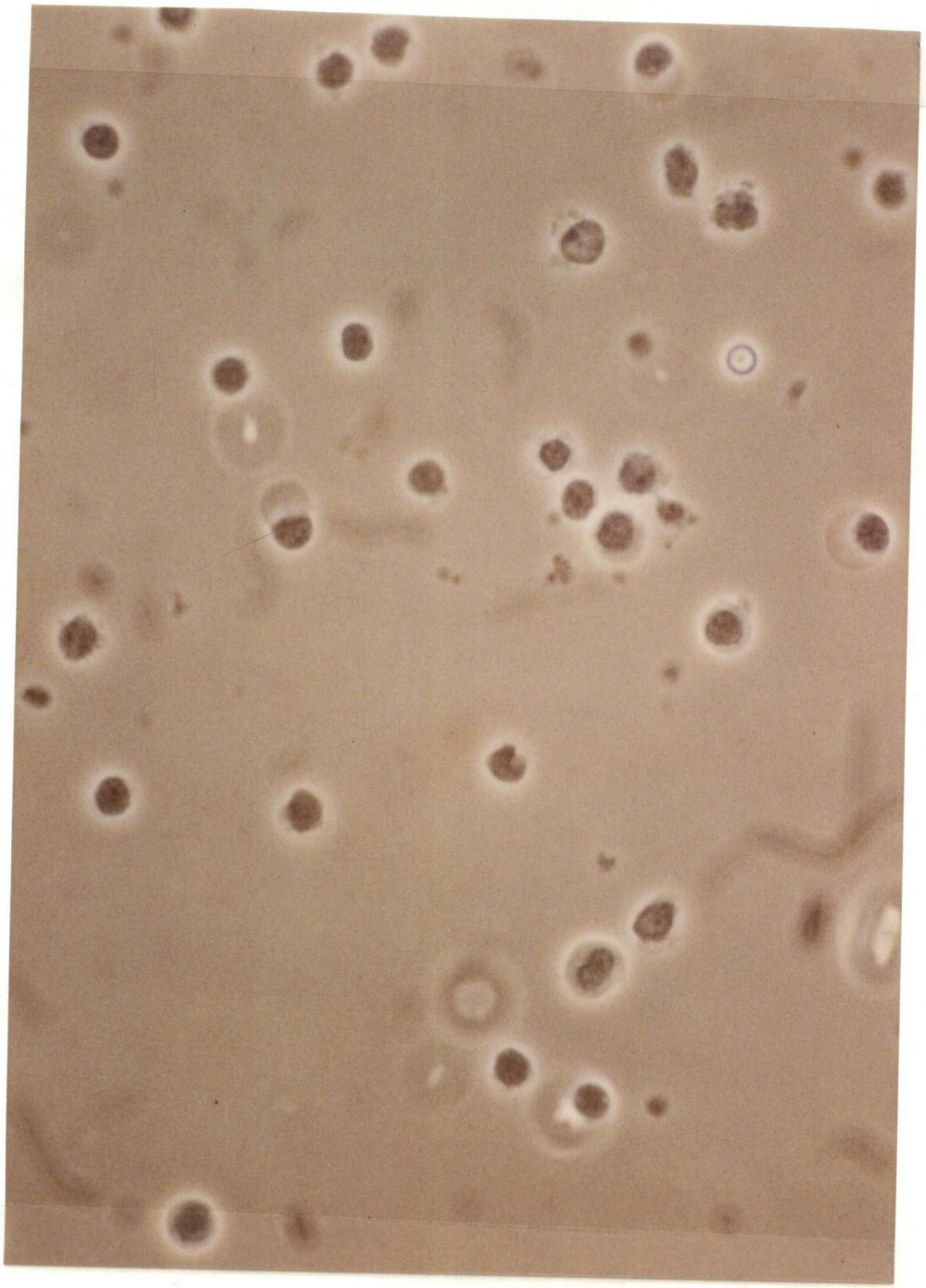


FIG 13 - B CELLS INCUBATED AT 85°C. NOTE THAT CELLS ARE INTACT.

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