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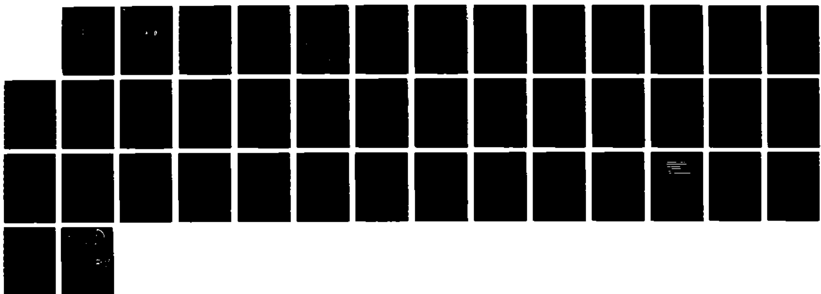

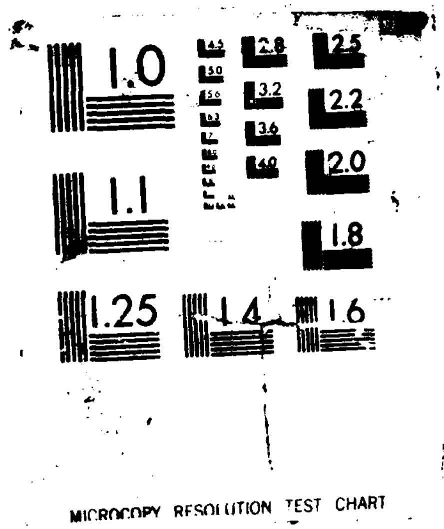

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AD-A184 469

PREVENTION OF CANINE GRAFT-VERSUS-HOST DISEASE (GVHD)

Fred Hutchinson Cancer Research Center
1124 Columbia Street
Seattle, WA 98104

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25 November 1986

Technical Report

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The work under the contract was aimed at the ultimate goal of solving the two major problems of marrow transplantation: "resistance" to grafts, and graft-versus-host disease. Work done towards these goals has included the establishment of a laboratory specialized in the production and characterization of murine monoclonal antibodies to canine mononuclear cells. Antibodies with a broad range of specificities have been generated and have served to establish a first breakdown of canine mononuclear cells into functional subsets, e.g., helper and suppressor cells. A large number of antibodies generated against cells from other species have been screened for cross-reactivity with canine cells with remarkable success, in particular antibodies against monomorphic framework structures of Ia. Antibodies, including cross-

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reactive ones, have been used in a model of autologous marrow transplantation, and strong evidence for the presence of Ia antigens on pluripotent hematopoietic stem cells has been obtained. Of interest was that Ia did not seem to be expressed on committed hematopoietic precursors. These findings are not only of great theoretical interest, but may also have practical implications with respect to positive sorting of pluripotent stem cells via immunoabsorbent columns. Furthermore, a technique of marrow incubation with a combination of antibodies and complement has been developed allowing T-cell removal by 4 dogs as determined by an assay involving limiting dilution. This technique will be used with appropriate antibodies in future marrow transplant experiments aimed at preventing graft-versus-host disease. Finally, ~~we have begun~~ making headway with the problem of "resistance" to grafts. The rate of hematopoietic engraftment in recipients of DLA-incompatible grafts was increased from 5 to 50% when recipients were treated in vivo with an antibody to a monomorphic Ia framework determinant (antibody 7.2). These exciting studies are being continued with other antibodies either given alone or combined with antimetabolites such as methotrexate administered after transplantation.

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PREFACE

Dogs are provided by the Center s canine breeding program and by licensed breeding facilities who have contracted to provide pedigreed dogs for research purposes. Dogs are housed in individual cages or runs according to the space recommendations of the "Guide for the Care and Use of Laboratory Animals". The facility is approved by the American Association for Accreditation of Laboratory Animal Care and is under regular inspection by the Department of Agriculture. Animal facilities are cleaned by trained personnel on a daily basis. Animals are fed once daily and have continuous access to fresh uncontaminated drinking water. All investigators are either physicians or veterinarians, and they are assisted by a chief animal technician who has been working in the canine research program for 25 years and has extensive experience in animal care and husbandry. All experimental dogs are seen twice daily by one of these individuals. Separate clinical charts are maintained on each dog. All animals that die unexpectedly or that are humanely euthanized undergo a careful autopsy with subsequent review of histologic specimens by trained pathologists. Carcasses are incinerated. Experimental protocols covering all the procedures performed are discussed at regular staff meetings and, after being approved by the principal investigator and senior staff members, are submitted to the Institutional Animal Care and Use Committee for approval.

All research activities and procedures described above are conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council.

TABLE OF CONTENTS

Section		Page
	Preface	iii
	List of Tables	iv
1	Graft Resistance	1
2	Production, Screening, and Characterization of Monoclonal Antibodies Against Canine Mononuclear Cells	9
3	Development of Techniques to Deplete T Cell From Bone Marrow and Assessment of its Effectiveness	12
4	Testing for Hemopoietic Toxicity of Monoclonal Antibodies Combined with Complement by In Vitro Culture of Committed Hemopoietic Precursors and Autologous Marrow Transplantation	15
	List of References	19

LIST OF TABLES

Table		Page
1	Engraftment rates in dogs given 900 rad total body irradiation and $\leq 4 \times 10^8$ marrow cells/kg.	21
2	Data on dogs given 900 rad of TBI, DLA-nonidentical unrelated marrow and postgrafting methotrexate	22
3	Dogs given 900 rad TBI and marrow grafts ($\leq 4 \times 10^8$ cells/kg) from DLA-nonidentical unrelated donors	23
4	Monoclonal antibodies generated against canine cells	24
5	Monoclonal antibodies generated against cells from other species that are cross-reactive with canine cells	25
6	Limiting dilution	26
7	Effect of treatment of marrow cells with McAb 7.2 and C' on GM-CFC and CFU-E	26
8	Autologous marrow (BMC) grafts following 9.2 Gy TBI (Experiment #123 D)	27
9	Autologous marrow (BMC) grafts following 9.2 Gy TBI (Experiment #123)	28
10	Autologous marrow (BMC) grafts following 9.2 Gy TBI	29

SECTION 1

GRAFT RESISTANCE

As outlined in the original proposal, resistance to DLA-incompatible marrow grafts has been defined as the major hurdle in the way to successful exploration of T cell-depleted marrow infusions for prevention of GVHD. Resistance is mediated by a relatively radiorefractory cell population. Preliminary in vivo and in vitro studies suggest that these cells are not classical T cells and that they express Ia-antigens as detected by the monoclonal antibody 7.2 directed at a monomorphic Ia framework structure. In view of the only partial success with in vivo treatment by antibody 7.2 in overcoming resistance (see below), we have concentrated on screening more anti-Ia antibodies for cross-reactivity with dogs and producing additional antibodies against cells presumed to be involved with mediating resistance.

1.1 SCREENING OF ADDITIONAL ANTI-IA ANTIBODIES.

A number of monoclonal antibodies in addition to antibody 7.2 have been obtained and results compared with those obtained with antibody 7.2. Antibody B1F6 (F13) (courtesy Dr. Alejandro) was raised against canine cells and recognizes a different epitope and molecules than 7.2 (1). Antibody ISCR3 (courtesy Dr. Sachs) was raised against murine Ia (2), and antibody HB10a (courtesy Dr. Clark) was raised against human Ia (18). These recognize a similar spectrum of canine cells as 7.2 by indirect immunofluorescence, but they appear to recognize different epitopes as assessed by an additive enzyme-linked immunoabsorbant assay

against canine lymphocytes (unpublished). Antibodies 332, 1-117, 7.12, 7.19, and 7.22 (courtesy Dr. Pesando, our own laboratory, unpublished) seem to recognize the same epitope as 7.2 but, other than some slight cross-blockage seen with antibody 1-117, there is no evidence of sharing of antigen binding sites between these antibodies and antibody 7.2. Antibody HB10a has already been used *in vivo* (see below). An additional antibody, P4.1 (Dr. Antonelli) cross-reacts with a smaller subpopulation of canine cells (unpublished). Biochemical studies (RIP-SDS-PAGE) have shown that antibodies 332, HB10a and ISCR3 identify typical p28-34 Ia-like antigens on canine cell lysates. Similarly, we confirmed that B1F6 identifies an Ia-like structure.

1.2 MONOCLONAL ANTIBODIES AGAINST "RADIORESISTANT CELLS" OBTAINED AFTER TBI.

A number of antibodies were produced against marrow and spleen cells obtained on day 6 after supralethal TBI. Day 6 was chosen in an attempt at having an enriched population of relatively radioresistant cells presumed to be involved in marrow graft rejection. Clones from fusions in which spleen cells were used as antigens are still being screened. Clones resulting from fusions where marrow cells were used as antigen yielded four interesting antibodies reported in the November 14, 1984 Progress Report and designated 16-169-S3, 16-134-S2, 16-134-S3, and 16-169-S5. These antibodies not only reacted strongly with normal marrow cells and cells of the stem cell rich fraction III of the albumin gradient, but also with marrow cells obtained on day 6 after TBI which, we hope, are the relatively radioresistant cells involved in graft rejection.

We are currently investigating these antibodies with regard to stem cell toxicity in the autologous marrow transplant model. If there is no pluripotent stem cell toxicity, we will examine whether these antibodies can be usefully added to the combination consisting of DLy6, E11, and DT2 to more efficiently remove T cells from marrow inocula. The usefulness of these antibodies for this purpose is suggested by their reactivity with peripheral blood lymphocytes, thymus cells, and IL-2 activated lymphocytes.

However, the major purpose of producing these antibodies is to examine them in experiments *in vivo* aimed at abrogating resistance as described below under Methods.

1.3 *IN VIVO* APPROACHES AT ABROGATING RESISTANCE TO DLA-NONIDENTICAL MARROW GRAFTS.

1.3.1 Methotrexate Facilitates Engraftment of DLA-Incompatible Unrelated Marrow.

The model is the same as that outlined in the original proposal. DLA-nonidentical donor-recipient combinations are used. Approximately 4 x 10^8 marrow cell/kg are infused after 900 rad of TBI. Dogs are given 0.4 mg of MTX/kg intravenously on days 1, 3, 6, 11 and once weekly after marrow transplantation. Marrow engraftment is assessed by rising and sustained white cell and platelet counts following the post irradiation nadir, the development of GVHD, marrow cellularity at autopsy, and cytogenetic analysis of marrow cells. Table 2 summarizes the results of 10 dogs so treated. Six showed evidence of sustained hemopoietic engraftment, in most of them accompanied by the development of rapidly fatal GVHD. Four

of the 10 failed to engraft. This compares to only one sustained engraftment among 20 dogs not given methotrexate (Table 1). Results suggest that cells involved in mediating resistance to mismatched marrow grafts remain metabolically active for sometime after 900 rad of TBI.

1.3.2 Facilitation of Engraftment of DLA-Nonidentical Marrow by Treatment of the Recipient with Monoclonal Anti-Ia Antibody.

As pointed out in the original contract proposal, our previous studies have led us to hypothesize that the radioresistant metabolically active non-T cells involved in mediating resistance are possibly macrophages or NK-like cells expressing Ia-like antigens. Therefore, we investigated the effect of a monoclonal anti-Ia antibody recognizing canine monocytes, a large population of lymphocytes and NK cells, on engraftment of DLA-nonidentical marrow. The model is again that described above. Table 3 summarizes the results (3 and unpublished). We concluded that antibody 7.2 facilitated engraftment of DLA-nonidentical marrow resulting in sustained takes in half of the dogs. Increasing the dose of antibody (group 3) or adding a second antibody HB10a (group 5) failed to increase or possibly even interfered with engraftment. Failure of engraftment in some dogs of group 2 and all dogs of group 3 may have been due to the presence after marrow infusion of "free" antibody, resulting either in inactivation of donor accessory cells required for engraftment or, perhaps, in stem cell toxicity (see below). Of interest are the results in dogs of group 6. In this group, since both antibody alone and MTX alone resulted in a 50-60% engraftment rate, we investigated whether the combination of anti-

body and MTX might increase the engraftment rate. The success in 4 of 4 dogs is encouraging but clearly needs to be confirmed in a larger series.

These data support the hypothesis that Ia-positive host cells are involved in graft resistance. The added beneficial effect of MTX post grafting indicates that the cells involved in resistance remain metabolically active, even after TBI. The establishment of an effective and reproducible method of overcoming resistance is important for marrow transplantation across major histocompatibility barriers, particularly if T-cell depleted marrow is used.

1.3.3 Studies of Radiolabeled Monoclonal Antibodies to Overcome Graft Resistance (in Part Supported by Grants CA 31787 and CA 18105).

While the observation that graft resistance can in part be overcome by treatment of recipients with an anti-Ia antibody is exciting, results have shown that this approach is not successful in all dogs. Since it is not known how the anti-Ia antibody works when administered _in vivo_, e.g. via cytolysis in the presence of canine complement, via blocking of antigenic epitopes or via removal of cells that are coated with antibody through the RES system, it is somewhat difficult to explain why only approximately half of the dogs have successful engraftment and the other half fails. However, it is reasonable to assume that destruction of all Ia-positive cells involved in resistance would increase the rate of success of DLA-nonidentical grafts. An attractive way of accomplishing this would be to conjugate antibody to radioactive isotopes

which, upon injection into the dog, would result in irradiation and destruction of antigen-expressing cells. We have therefore initiated studies of antibody isotope conjugates in dogs. The first antibody studied has been the anti-Ia-like antibody 7.2 (16). In these preliminary experiments, iodinations were performed with iodogen and radioiodine ($NA^{125}I$ or $NA^{131}I$). Unbound iodine was removed by passage over a Sephadex PD-10,G25 column. Avidity after labeling was determined from Scatchard plots of the binding of labeled antibody to relevant target cells.

We injected 1 mg/kg of ^{131}I-labeled 7.2 along with 1 mg/kg of ^{1-25}I-labeled isotype matched irrelevant control antibody into 3 normal dogs. Dogs were sequentially scanned in a Gamma camera for 24-48 hours and then killed to determine the concentrations of ^{131}I and ^{125}I in various organs. By establishing the correlation between the activity measured by scanning over the organ in question with the actual concentration of the radionuclide found at autopsy, kinetics of the antibody conjugate over time were determined in the liver, spleen, and marrow. In our studies, more than 90% of both isotopes were protein-bound (TCA precipitable) and cell-free. Our studies showed that only in the spleen, lymph nodes and marrow, the concentration of ^{131}I was higher than in the blood, while in no organ was there concentration of the irrelevant antibody greater than blood. The sequential scanning, combined with the values found at autopsy, allowed us to construct biodistribution curves. By integrating the areas under the curves, based on a dose of 1000 rad delivered to the blood, the cumulative dose delivered to spleen, bone marrow, and liver can be estimated as spleen = 3,230, bone

marrow = 1,630, liver = 1,080. These studies suggest that the use of Ia-labeled monoclonals should allow us to increase the radiation dose to the spleen by 3.2 times and to marrow by 1.6 times without increasing the dose to any other organ.

In these studies we noted an early hepatic activity followed by a subsequent fall. This represents an interesting potentially difficult problem in that hepatic toxicity is not infrequently seen following marrow transplantation using external beam TBI and we would like to avoid undue hepatic irradiation. In order to understand this early uptake, we scanned a dog after injection of an irrelevant isotype-matched monoclonal conjugated to ^{131}I. In this study, the rapid hepatic uptake did not occur, demonstrating that this is an antigen-specific phenomenon. Further _in vivo_ and _in vitro_ studies, including examination of liver tissue using immunoperoxidase techniques, corroborated that Kuppfer cells are Ia-positive. This suggests that the early uptake of radiolabeled 7.2 is due to direct antigen-specific binding. We are now exploring whether this problem can be bypassed by infusing "cold" 7.2 antibody prior to treatment with labeled antibody thereby decreasing hepatic accumulation of labeled antibody.

These studies, while preliminary, are exciting and will be expanded. The initial studies of screening of various antibodies will continue to involve radioactive iodine because of the relative simplicity of this approach and the ability of scanning through a readily available gamma camera. Our ultimate plan is to extend these studies to the use of positron-emitting isotopes conjugated to monoclonal antibodies which will allow for much more accurate

determination of the 3-dimensional distribution of a labeled antibody over time through positron emission tomography. Preliminary studies have been initiated with members of the Nuclear Medicine Division of the Department of Medicine here at the University of Washington (Drs. Krohn and Nelp).

SECTION 2

PRODUCTION, SCREENING, AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST CANINE MONONUCLEAR CELLS

We have pursued two general approaches at expanding the number of monoclonal antibodies available to us. One involved immunization of mice with various canine cells (see also the preceding section on graft resistance) and one consisted of testing antibodies originally produced against cells from other species for cross-reactivity for canine cells.

Figure 1 summarizes an earlier attempt at assigning specificities of monoclonal antibodies for canine lymphocytes and monocytes reported in greater detail in previous progress reports and the previous contract (4-9).

2.1 ANTIBODIES PRODUCED AGAINST CANINE CELLS.

Table 4 summarizes some of the newer antibodies. Neither the antigens recognized by these antibodies nor the functional properties of the cells on which antigens are expressed have as yet been completely characterized.

2.2 SCREENING OF ANTIBODIES DIRECTED AGAINST CELLS FROM OTHER SPECIES.

A large panel of murine monoclonal antibodies directed against cells from man and other animal species have been tested for cross-reactivity against canine cells. Table 5 summarizes some of the potentially interesting cross-reactive monoclonal antibodies.

2.2.1 Antibodies from the T-cell Monoclonal Workshop in Paris, 1982.

Seventy-seven such antibodies were tested. Of these, 14 showed some cross-reactivity. They were screened against canine peripheral blood lymphocytes, thymocytes and bronchoalveolar macrophages. None reacted with macrophages. Six showed reactivity with thymocytes, of which 3 recognized an OKT6-like antigen in man, an antigen confined to the thymus and dendritic cells in humans. In dogs they all recognized a thymus-specific antigen, and studies on dendritic cells are in progress. The only antibody showing reactivity with thymocytes and peripheral blood lymphocytes was T811 (Dr. Rieber). This antibody recognizes in man a suppressor/cytotoxic cell population and also labels NK cells. In dogs it recognizes an antigen expressed on 19-27% peripheral blood mononuclear cells, 20-30% peripheral blood Thyl-positive T lymphocytes, 40% of puppy thymocytes, 35-45% of alloantigen activated peripheral blood lymphocytes and 100% of peripheral blood mononuclear cells from a dog with T-cell acute leukemia (8). The antigen was not expressed on peripheral Thy-1 negative cells, bronchoalveolar cells, or null-type acute leukemia cells. The antibody inhibited cell-mediated lympholysis in the absence of complement, while antibody F3-20-7 directed at canine Thy-1 antigen did not. There was no antigenic modulation. We have not been able to precipitate a corresponding cell surface antigen by RIP techniques.

2.2.2 Ia-Like Antibodies.

Numerous Ia-like antibodies have been screened. Antihuman monoclonal 7.2 has been extensively studied in the dog as outlined

in previous progress reports. Further information is given in the section above under "Graft resistance".

2.2.3 Other Antibodies.

Four of 9 antibodies against the ubiquitous 200,000 molecular weight antigen of man cross-reacted in various proportions with dog lymphocytes. Functionally, these antibodies are capable of blocking NK recognition and effect in man. Antibody 13.1 (Dr. Newman) failed to block NK activity in the dog (unpublished). In addition, the molecular weight of the antigen recognized in the dog by SDS page was a dimer of 47,000 and 52,000 daltons rather than a tetramer centered on 200,000. Other antibodies examined were 13.4, 13.6, and 9.4 (the latter from the laboratory of Dr. Hansen) (10). All recognize approximately 20% of canine peripheral blood lymphocytes, a small population of thymocytes, and cells from a dog with T-cell ALL.

Another interesting antibody is 60.3, an IgG2a produced by Dr. Beatty (19). This antibody is a "common leukocyte" antibody which blocks cytotoxic cells, NK cells, and lymphocyte transformation. In man, it recognizes a dimer of 150 and 95 k and in dogs it recognizes a dimer of 97 and 170, with an additional band at 180 k. It is reactive with most canine hemopoietic cells and blocks CML reactivity. This would seem to be an interesting antibody for antibody isotope conjugate studies.

SECTION 3

DEVELOPMENT OF TECHNIQUES TO DEPLETE T CELL FROM BONE MARROW AND ASSESSMENT OF ITS EFFECTIVENESS

Experiments were carried out to optimize conditions for treatment of marrow with antibodies and complement. Previously, unseparated marrow was treated, with difficulty in assessment of residual cellular populations. Best yields of nucleated cells and least erythrocyte and granulocyte contamination were obtained by density gradient separation of highly diluted (x5 - x7) marrow over Ficoll Hypaque (S.G. 1.077). Final cell yields were 25-50% of initial nucleated cell count with the major reduction in the granulocyte fraction. Red cell removal was proportional to the degree of dilution. After labelling with antibody, treatment with two cycles of complement led to increased cell kill whereas further complement treatments did not. Experiments were then directed at assessing residual populations of T cells. Three approaches were used:

3.1 DIRECT ASSESSMENT USING THE FACS AND LABELING WITH F3-20-7 AGAINST THY-1.

Add-back techniques have shown us that a reduction of Thy-1 positive cells by 2 logs can be recognized with this approach.

3.2 PHA-STIMULATED CULTURES.

Marrow is cultured for 7 days in the presence of PHA after treatment with monoclonal antibody and complement. Subsequently, the cultured cells are being labeled with anti-Thy-1 and are evaluated on the FACS.

With this technique, a reduction in T cells by 3 logs can be recognized.

3.3 LIMITING DILUTION. (12)

Ficoll Hypaque separated marrow cells are treated with monoclonal antibody and complement. Then, 10^5 marrow cells in a volume of 100 uL RPMI containing 20% dog serum are plated into each well of a microtiter plate with PHA present in a concentration of 2 ug/ml. Zero, 1, 2, 4, 8, 16 lymph node lymphocytes suspended in 100 uL of RPMI+20% dog serum are then added to consecutive wells. Every second day cultures are fed with RPMI+20% dog serum + 5% IL2 (cellular products) in a total volume of 100 uL/well. On the 18th day of culture 1 uCi of ^3H thymidine is added to each culture well. After 4 hours exposure, cells are harvested with a Mash harvester and the amount of incorporated ^3H thymidine is determined with a scintillation counter. Wells without added lymph node lymphocytes serve as the background: the ^3H thymidine incorporation of wells so treated is, on the average, 600 cpm $\pm$ 180 CPM (3 standard deviations). Values above 780 cpm are counted as "hits". The percent of nonhits decreases as the number of added lymphocytes increases (Table 6). If the percent of nonhits is plotted on semi-log paper against the number of added lymphocytes, a straight line is found. The point where the line crosses the Y axis indicates the number of lymphocytes which should have been removed to achieve 100% nonhits. In our studies, this number is 4.4, i.e. we can still find 4.4 residual T cells among 100,000 cultured marrow cells. With this technique of limiting dilution, therefore, a reduction of T cells by 4.5 logs can just be recognized.

Treatment of marrow cells with DLy-1 was found to remove 4 logs of T cells. With a combination of DLy-6, E11, and DT-2, we have achieved a

reduction by 1 log. Adding WIG4 to this combination results in a reduction by 2 logs. These studies are being continued with the addition of further antibodies to the combination, in particular, W1D10, C3D1, and newer antibodies currently being screened.

SECTION 4

TESTING FOR HEMOPOIETIC TOXICITY OF MONOCLONAL ANTIBODIES COMBINED WITH COMPLEMENT BY IN VITRO CULTURE OF COMMITTED HEMOPOIETIC PRECURSORS AND AUTOLOGOUS MARROW TRANSPLANTATION (17)

Traditionally, assays for committed hemopoietic progenitors such as CFU-E and CFU-C have been used to evaluate toxicity of *in vitro* treatment of marrow. The dog offers the possibility to investigate toxicity on pluripotent stem cells via the approach of autologous marrow transplantation after otherwise lethal TBI. Any antibody that has been of interest for studies of marrow incubation has been tested both *in vitro* with regard to effects on committed hemopoietic precursors and *in vivo* with regard to an effect on marrow engraftment (see original contract proposal, the various progress reports, and above).

An important observation has been the finding that an Ia-like antigen, as detected by monoclonal antibody 7.2 resides on pluripotent stem cells but not on committed progenitors. Table 7 gives an example of the lack of effect of treatment of marrow cells with monoclonal antibody 7.2 and complement on GM-CFC and CFU-E. In contrast to the *in vitro* findings, the results of the *in vivo* experiment #123 D as shown in Table 8 indicate that treatment of marrow with 7.2 and complement results in failure of sustained engraftment in animals so treated. The same table shows that neither Ficoll Hypaque separation nor treatment with complement alone impair the ability of marrow for sustained hemopoietic reconstitution. The findings could be explained in two ways: 1) pluripotent

hemopoietic stem cells of the dog express Ia antigens while committed hemopoietic stem cells are Ia negative; 2) pluripotent and committed hemopoietic stem cells are Ia negative, but, for a marrow graft to take, Ia-positive accessory cells are required. To clarify these possibilities, lethally irradiated dogs were given marrow which was treated with antibody 7.2 and complement, and additionally stem cell-free peripheral blood leukocytes obtained from fraction V (± fraction IV) of an albumin gradient. Table 9 shows the data in 8 dogs so treated. All failed to show sustained engraftment although adequate numbers of mononuclear cells were infused. A control dog receiving cells from fraction V alone also failed to show hemopoietic engraftment. These results are consistent with the hypothesis that pluripotent stem cells of the dog are Ia positive (as determined by antibody 7.2) in contrast to committed hemopoietic precursors. The transient engraftment seen and the minimal foci of hemopoiesis seen at autopsy in some of the dogs might be explained by proliferation of cells originating in the committed precursor compartments.

In order to obtain positive proof that pluripotent hemopoietic stem cells express Ia, we carried out the experiment shown in Table 10 (123J) where bone marrow cells were separated with Ficoll Hypaque and then split into fractions using the albumin gradient. Cells of the stem cell-rich fraction III were stained with antibody 7.2 and FITC with subsequent positive selection on the FACS 440. After a 12-14 hour cell sort, a relatively small number of 7.2-positive cells was obtained (Table 10) and then injected intravenously into the lethally irradiated autologous recipients. All grafts failed. To further increase the

number of 7.2-positive cells in this experiment, cells of fraction III were treated with a combination of monoclonal anti-T cell antibodies DLy-6, E11, and DT-2 plus complement (dog C135); this increased the cell yield somewhat. Nevertheless, this dog also failed to show sustained engraftment. Results from this experiment could be explained in three ways: 1) the number of transplanted 7.2-positive marrow cells was too small for sustained engraftment; 2) fraction III of the albumin gradient does not contain hemopoietic stem cells; 3) the cells coated with the antibody 7.2 are removed through the RES system of the recipient after infusion. Possibility 2 was excluded: Dogs C116 and C141 received marrow cells from fraction III of the albumin gradient and both showed sustained engraftment. Possibility 3 was also excluded: Dogs C124 and C117 received marrow cells coated with monoclonal antibody 7.2 without the addition of complement and both showed sustained engraftment. By exclusion, therefore, we are left with the hypothesis that, while pluripotent hemopoietic stem cells express antigen detected by antibody 7.2, the number of 7.2-positive cells obtained through the cell sorter was too small for sustained engraftment.

In order to obtain positive proof for the presence of Ia antigen on pluripotent stem cells we will use a technique of cell affinity chromatography which promises much higher yields.

As explained above, similar studies with autologous marrow grafts were carried out to investigate the safety of T cell removal from marrow through assessing the ability of a marrow so treated to show sustained and long-term hematopoietic engraftment. Thus, the technique of autolo-

gous marrow transplantation combined with the committed precursor assays are excellent tools to obtain knowledge regarding the presence or absence of certain monoclonal antibody defined antigens on pluripotent stem cells. These studies might result in the development of a technique allowing us to positively select for stem cells. They also might help us gaining insight into the role, if any, of lymphoid "accessory" cells in sustaining hemopoietic grafts. The studies of autologous marrow transplantation with T-cell depleted marrow are going to be of importance to interpret future results with transplants of incubated marrow across minor and major histocompatibility barriers.

SECTION 5

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Table 1. Engraftment rates in dogs given 900 rad total body irradiation and $\leq 4 \times 10^8$ marrow cells/kg.

Donor-Recipient Combination	Number of Dogs Studied	% with Sustained Graft	% with Rejection
Littermates:			
DLA-identical	15	93	7
DLA-nonidentical	12	8	92
Unrelated:			
DLA-identical	27	52	48
DLA-nonidentical	20	5	95

Table 2. Data on dogs given 900 rad of TBI, DLA-nonidentical unrelated marrow and postgrafting methotrexate.

Dog No.	Marrow Cells infused ($\times 10^8$/kg)	Peak WBC ($\times 10^9$)[1] (day)/% granulocytes	Evidence of GVHD[2]		Marrow cellularity (% of normal at autopsy	Sustained Engraftment	Survival (days)	Cause of death
			clinical	histological				
C63	4.0	0.143(d.7)/15	No	No	<5	No	9	Septicemia, pneumonia (E. coli)
B786	2.3	0.53(d.1)/88	No	No	<5	No	16	Hemorrhage
B757	1.9	0.64(d.11/89	No	No	<5	No	16	Septicemia (E. coli)
B891	4.0	0.145(d.20)/61	No	No	<5	No	20	Sacrificed, Septicemia (Klebsiella oxytoca)
C62	3.9	0.185(d.7)/90	Yes; E	Yes; L	30	Yes	9	Pneumonia (E. coli)
B743	3.7	7.5(d.18)/71	Yes; S,L,E	Yes; S,L,G	100	Yes	19	GVHD, Septicemia (Diphtheroids)
B746	1.3	5.8(d.18)/80	Yes; S,L,E	Yes; S,L	100	Yes	19	GVHD, Septicemia, pneumonia (E. coli)
B740	4.0	8.8(d.21)/89	Yes; S,E	Yes; S,L	100	Yes	22	GVHD, Septicemia (E. coli)
B790	4.0	5.1(d.32)/76	Yes; S,L,E	Yes; S,L,G	100	Yes	34	GVHD Hepatic failure
B831	3.6	5.1(d.74)/87	No	No	90	Yes	82	Malnutrition, Cryptosporidium enteritis

[1] Peripheral white blood cell count per liter.

[2] Graft-versus-host disease; E=eyes, S=skin, L=liver, G=gut.

Table 3. Dogs given 900 rad TBI and marrow grafts ($\leq 4 \times 10^8$ cells/kg) from DLA-nonidentical unrelated donors[1].

Group	Treatment Regimen		Number of Dogs		
			Studied	With Engraftment	With Rejection
1	TBI		21	1	20
2	TBI, mcAb 7.2, 0.2 mg/kg	d. -1 to 3	6	3	3
3	TBI, mcAb 7.2, 0.4 mg/kg	d. -1 to 3	3	0	3
4	TBI, mcAb 7.2, 0.2 mg/kg	d. -5 to 0	6	3	3
5	TBI, mcAb 7.2, 0.2 mg/kg mcAb HB10a, 0.2 mg/kg	d. -5 to 0	2	1	1
6	TBI, mcAb 7.2, 0.2 mg/kg MTX, 0.4 mg/kg	d. -5 to 0 d. 1,3,6,11	4	4	0

[1] TBI = total body irradiation; mcAb = monoclonal antibody; d. = day (relative to day of TBI and marrow transplantation); MTX = methotrexate, given intravenously. Both mcAb 7.2 and HB10a recognize a framework determinant of Ia-like antigens but differ in their binding sites.

Table 4. Monoclonal antibodies generated against canine cells.

	Ig Subclass[2]	Mean Percent Reactivity[1]					Antigen(s)[3]
		PBMC	monocytes	granulocytes	bone marrow	thymocytes	
DLy1	IgG2b	57-62[4]	69	73	31-38[4]	20	p213
Dt2	IgG2a	23-27[4]	17	12	12-23[4]	10	p70
DLy6	IgM	22-35[4]	8	5	14-15[4]	2	p60
E11	IgG3	16-23[4]	30	ND[5]	14-50[4]	4	ND
A5	IgG2b	75[6]	ND	ND	ND	85[6]	p96
Wig4	IgG1	10	90	ND	6-7[4]	0[6]	ND
3G10	IgM	33	ND	ND	ND	ND	p38
1H3	IgG2a	21	50[6]	ND	18	7[6]	p26
W1D10	IgG1	6	ND	ND	3	10	p39
16-134-S2	IgG2b	68	70	83	69	31	ND
16-134-S3	IgG2b	64	75	81	56	32	ND
16-151-S1	ND	7	60	71	21	6	ND
16-151-S3	ND	37	60	68	64	10	ND
16-169-S3	IgG1	71	50	82	57	33	ND
16-169-S5	IgG1	72	85	79	59	19	p95
B1F6 (F13)[7]	IgG2	78	ND	ND	ND	ND	p29,34
F3-20-7 (anti-Thy1)[8]	IgG1	45-51[4]	59	9	12-13[4]	43	ND

[1] Determined by cytofluorometry (FACS II and IV) using cells from 5 to 10 different dogs.
[2] Evaluated by antimouse Ig isotyping reagents (Tago, Inc).
[3] Evaluated by SDS-PAGE radioimmunoprecipitation.
[4] Data generated from 2 different studies of 5 to 10 dogs each.
[5] ND = not done.
[6] Data generated with cells from less than 5 dogs.
[7] Originally generated and described by Alejandro et al (1).
[8] Originally generated and described by McKenzie and Fabre (13).

Table 5. Monoclonal antibodies generated against cells from other species that are cross-reactive with canine cells.

	Immunizing Species[1]	Specificity	Ig subclass[2]	Mean Percent Reactivity[3]		Antigen(s)[4] (dog)
				PBMC	bone marrow	
7.2[5]	H	DR	IgG2b	50	14-20[6]	p29,34
ISCR3[7]	M	IE	IgG2	46	16	p29,34
HB10a[8]	H	DP	IgG2	73	ND	p29,34
60.3[9]	H	leukocyte	IgG2a	51	29	p97,170,180
60.5[9]	H	class I	IgG3	57	ND	p45
T811[10]	H	T suppressor	IgG2	14-27[6]	11	ND
40.1[11]	H	DR(MT)	IgG2	37	ND	ND
40.3[11]	H	DR,DQ	IgG2	42	ND	ND
9.4[5]	H	pan lymph.	IgG2a	5	11	ND
H58A[12]	P	class I	IgG2a	86	ND	ND
H34A[12]	B	class II	IgG2	56	ND	ND
H42A[12]	P	class II	IgG2	48	ND	ND
TH81A[12]	P	DQ	IgG2	52	ND	ND
I-3.1e[13]	H	DP	IgG2	18	6	p29,34
gp24[13]	H	leukocyte	IgG2	15	11	ND

1 Abbreviations: H = human, M = mouse, P = pig, B = bovine, ND = not done.
2 Evaluated by antimouse Ig isotyping reagents (Tago, Inc.).
3 Determined by cytofluorometry (FACS II and IV) using cells from 5 to 10 different dogs.
4 Evaluated by SDS-PAGE radioimmunoprecipitation.
5 Antibodies originally described by Hansen et al (10).
6 Data generated from 2 different studies (details in reference 2).
7 Antibody originally described by Watanabe et al (2).
8 Antibody developed by E. Clark (18).
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10 Antibody originally described by P. Rieber et al (11).
11 Antibodies developed by M. Pierres (unpublished).
12 Antibodies originally described by Davis et al (14).
13 Antibodies developed by J. Pesando (unpublished).

Table 6. Limiting dilution.

"threshold" = mean + 3SD = 780

lymphocytes	hits	nonhits	% nonhits
0	6	10	62.5
1	3	13	81.25
2	5	7	58.3
4	4	8	66.6
8	7	5	41.7
16	9	3	25.0

Table 7. Effect of treatment of marrow cells with McAb 7.2 and C' on GM-CFC and CFU-E.

	GM-CFC		CFU-E	
	Per 10^5 BMC		Per 10^5 BMC	
Exp. No.	Pre 7.2	Post 7.2	Pre 7.2	Post 7.2
1	112 ± 12	215 ± 26	27 ± 24	96 ± 108
2	553 ± 91	502 ± 51	432 ± 54	615 ± 84
3	845 ± 64	1989 ± 108	42 ± 21	117 ± 24

Table 8. Autologous marrow (BMC) grafts following 9.2 Gy TBI. (Experiment #123 D).

Dog Number	Treatment of BMC	No of BMC Infused ($x10^{-8}$/kg)	%7.2+ Cells Pre Treatment	%7.2+ Cells Post Treatment	%HB10a+ Cells Pre Treatment	%HB10a+ Cells Post Treatment	Sustained Graft	Temporary Engraftment	Histo. Signs Of Engraftment @ Autopsy	Survival (Days)
B 994	7.2+C'	0.6	1	0	11	0	No	Yes	No	21
B 899	7.2+C'	0.6	4	0	12	0	No	Yes	No	22
C 32	7.2+C'	1.4	3	1	39	4	No	Yes	No	19
C 72	7.2+C'	0.2	8	0	32	7	No	No	No	17
C 101	none	1.7	--	--	--	--	Yes	--	--	>60
B 900	*none*	0.6	--	--	--	--	Yes	--	--	>60
C 46	C'	1.3	--	--	--	--	Yes	--	--	>60
C 142	C'	0.8	--	--	--	--	Yes	--	--	>60

Table 9. Autologous marrow (BMC) grafts following 9.2 Gy TBI. (Experiment #123).

Dog Number	No of BMC Infused ($x10^{-8}$/kg)	%7.2+ Cells Pre Treatment	%7.2+ Cells Post Treatment	%HB10a+ Cells Pre Treatment	%HB10a+ Cells Post Treatment	Cell No. of Frac V ($x10^{-8}$/kg)	Sustained Graft	Temporary Engraftment	Histo. Signs Of Engraftment @ Autopsy	Survival (Days)
C 110	0.5	11	0	15	0	0.06	No	No	Yes	8
C 114	0.16	7	0	6	0	0.07	No	Yes	No	18
C 126	0.4	16	0	17	0	0.12	No	Yes	Yes	11
C 111	0.2	28	2	43	0	0.10	No	Yes	Yes	11
C 45	0.4	9	0	13	0	0.04	No	No	Yes	12
C 58	0.4	35	0	12	0	0.14	No	Yes	Yes	21
C 122	0.3	25	2	19	0	frac V: 0.2 + frac IV: 0.3	No	Yes	Yes	14
C 115	0.8	21	1	5	0	frac V: 0.2 + frac IV: 0.2	No	No	Yes	9
C 86	--	--	--	--	--	0.04	No	No	No	9

Table 10. Autologous marrow (BMC) grafts following 9.2 Gy TBI.

Dog Number	No. of BMC Infused ($x10^{-8}$/kg)	Method to Pre-enrich for Stem Cells Before Sort	Sustained Graft	Temporary Engraftment	Histo. Signs of Engraft-ment @ Autopsy	Survival (days)
Experiment 123 J (Cell Sort for 7.2^+ BMC)						
C 134	0.007	Fraction III	No	Yes	Yes	11
C 108	0.03	Fraction III	No	No	No	7
C 135	0.05	Fract III, treated with DLy-6, E11, DT-2 and C'	No	No	Yes	9
Experiment 123 J (BMC Coated with 7.2 without C')						
C 124	1.0		Yes	--	--	>60
C 117	1.0		Yes	--	--	>60
Experiment 166 II						
C 116	0.3		Yes	--	--	>60
C 141	0.25		Yes	--	--	>60

Antibody
Lymphocytes
T Cells
B Cells
Mono-cytes
DLy-1
DLy-6
Thy1[2]
DT2 (helper)
E11[1] (suppr.)
7.2[3] (Ia)
α dog Ig[4]

1 = laboratory number of antibody-producing clone;
2 = a murine monoclonal antibody recognizing a Thyl-like antigen on canine cells (provided by Drs. McKenzie and Fabre);
3 = an antibody recognizing Ia-like antigens;
4 = polyclonal $F(ab')_2$ fragments of rabbit IgG specific for canine immunoglobulin (Cappel Laboratories).

Figure 1. Assignment of antibody specificities.

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