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CHARACTERIZATION OF A SERUM INHIBITOR OF MLC REACTIONS

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SUMMARY

Serum from a healthy multigravida female was found to inhibit most MLC combinations tested. Attempts to characterize the inhibitory factor have revealed that it is not lymphocytotoxic, destroyed by heat or directed against HL-A specificities. The inhibitory activity segregates with the 7S IgG fraction, is effective in very small concentrations and consistently fails to inhibit the MLC combinations between cells from the serum donor and those from one other unrelated individual. The blocking effect on MLC occurs during the first 6–9 hr of incubation. Inhibitory activity persists in the donor's serum 16 months after delivery of her last child. An attempt to utilize the inhibitory serum to minimize an expected graft-versus-host (GVH) reaction following bone marrow transplantation to the serum donor's daughter with marrow from the child's father resulted in neither GVH nor a 'take' of the graft. The possible effects of the serum on the grafted cells are discussed. While a recognition site for the inhibitory effect remains unknown, it appears to involve a T-cell receptor.

INTRODUCTION

During the process of selecting a potential donor for bone marrow transplantation of a child with acute lymphocytic leukaemia, it was noted that in one-way mixed leucocyte cultures (MLC) the leucocytes of the patient's mother did not respond to leucocytes of any of her children when autologous plasma had been used to support cell growth. A subsequent study clearly showed that when AB or pooled serum was employed, this woman's leucocytes responded normally to leucocytes of her children and unrelated persons. Further, it could be shown that her serum or plasma inhibited *in vitro* leucocyte responses of most other persons as well. We attempted to characterize this inhibitory activity and its mode of action on leucocytes.

MATERIALS AND METHODS

Our techniques for testing lymphocyte responses to phytohaemagglutinin (PHA) and to allogeneic cells (MLC) have been previously described (Gatti, Garrioch & Good, 1970). Culture medium: Minimal Essential Medium No. 138 (Grand Island Biological Co.) supplemented with glutamine (2 mM/ml), heat-inactivated pooled human serum, penicillin

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(100 U/ml) and streptomycin (100 μg/ml). Our 'pooled serum' is derived from healthy young male donors who have never received a blood transfusion. One batch of pooled serum is usually sufficient for 3 months of laboratory studies. Six different batches of pooled serum were used during the course of these investigations. In early experiments designed to test the effects of JH sera on MLC responses, 10% of either pooled or JH serum was added to each culture. In later experiments, all cultures were supplemented with 7·5% pooled serum: test fractions or similarly-treated controls were then added in 5% concentrations. Once these sera had been manipulated and/or fractionated, they often no longer supported growth and thus could no longer be considered as 'serum'. All dilutions of JH sera were made with pooled serum. Except where specifically stated, both JH and pooled sera were heat-inactivated (56°C for 30 min) before testing. Lymphocyte responses were assessed by uptake of radioactive thymidine (specific activity 5·6 Ci/mM) as measured in a liquid scintillation counter and expressed as the average of triplicate cultures in cpm.

Sera from JH were obtained in several ways and on numerous occasions beginning 3 months after delivery of her last child. Most studies described herein were performed using a single large blood sample collected, through a calcium-removing resin, in February 1971 (i.e. JH 2.71). The fibrin was removed from individual aliquots of this specimen by adding calcium chloride (100 μl/10 ml) prior to testing. Other blood samples were collected into heparin (25 U/ml) so that JH's cells could also be harvested. Periodically, in order to document the continuing presence of the inhibitor, small blood samples were drawn without any anticoagulant. Inhibitory activity was approximately the same in all of these specimens. Although not completely accurate, all of the specimens will hereafter be referred to as JH 'serum'. The serum administered ultimately to the daughter of JH, prior to and following bone marrow transplantation, was collected in the hospital Blood Bank through a calcium-removing resin and stored in accordance with recommended blood-banking procedures.

JH is a 35-yr-old para vi/gravida vi/abortus O female who is in excellent health. She has a twin brother. Her last child was born in July 1970. There is no known consanguinity between JH and her husband. JH has never received a blood transfusion.

Cell elutions were performed by two methods. The cells used were from the husband of JH and had been previously incubated with JH serum (50 ml packed cells/2 ml JH serum) for 1 hr with rocking. Half of the cells were incubated at 4°C (I) while the other half were incubated at 37°C (II). The cells were then washed four times with MEM and further subdivided into two equal aliquots (A and B). Aliquots IA and IIB were heated to 56°C for 10 min, as described by Landsteiner & Miller (1925) with continuous shaking. They were centrifuged for 10 min in pre-heated cups (56°C). The supernatant was dialysed for 24 hr against MEM and then tested for inhibitory activity. Aliquots IA and IB were frozen and thawed twice. 10 ml of cold 50% ethanol was added. The cells were then frozen overnight. The thawing slush was centrifuged (3000 rpm for 5 min), the pellet saved, washed with abundant amounts of distilled water, resuspended in the original volume of MEM and incubated for 1 hr at 37°C. After centrifugation, the supernatant was dialysed for 24 hr against MEM and then tested for inhibitory activity. (See Section 6 of Results.)

RESULTS

1. *Inhibitory effect of sera from JH on MLC responses*

Serum derived from JH was inhibitory to MLC responses of autologous leucocytes

TABLE 1. Inhibitory effect of sera JH 10.70 and JH 12.70 on MLC responses (cpm H_3T)

MLC* (haptolypes)	HL-A types	Experiment 1		Experiment 2	
		Pooled serum	JH 10.70	Pooled serum	JH 12.70
1. JH × JH_m (c/d)	5, 11	792	798	535	357
2. JH × $Husb_m$ (a/b)	5 × 12	19234	8900	23197	5056
3. JH × Son_m (a/d)	5 × 11, 12	21841	2164	25713	4694
4. JH × Dau_m (b/c)	5 × 5, 12	28561	7543	25472	5841
5. JH × UNR-1_m	5 × 2, 3	37185	10127	49656	6746
6. JH × UNR-2_m	5 × 2, 5, 10	20915	21703	22283	19043
7. UNR-1 × $Husb_m$	2, 3 × 12	32428	4239	33193	501
8. UNR-2 × $Husb_m$	2, 5, 10 × 12	10467	7076	12403	271
9. Husb × JH_m	12 × 5, 11			15329	552

UNR = unrelated to JH or to her husband. Husb = husband of JH.

* Sub_m denotes mitomycin-treated cells.

against those of her husband, her children and most unrelated persons (Table 1). Her serum also inhibited MLC responses of the leucocytes of most individuals tested. This inhibition did not appear to be related to HL-A types or to presumed haplotypes within Family H (discussed in detail below). The response of leucocytes against the mitomycin-treated leucocytes of UNR-2 was not inhibited by JH sera. This finding was consistent in numerous experiments performed over an 11-month period. It appeared that the inhibitory effect could be directed against either the stimulating or the responding cells.

2. *Heat-inactivation*

JH sera were inhibitory to appropriate lymphocyte cultures both before and after heat-inactivation. Most studies were performed with heat-inactivated JH serum.

3. *Cytotoxicity*

Serum specimens derived from JH at various times after delivery of her sixth child were not cytotoxic to lymphocytes of her husband or to those of an eighteen-person cell panel containing various HL-A specificities. These studies were performed using either rabbit or human complement.

Culture of leucocytes from the serum donor's husband (DH) with medium containing JH serum, under conditions simulating mixed leucocyte cultures, failed to show significant cytotoxicity during the first 5 days of culture, as assessed by trypan blue exclusion of mononuclear cells. By the sixth and seventh days of incubation, a slightly increased percentage (7·5% and 11% respectively) of cell death was noted in cultures containing the husband's cells with JH serum as compared to controls.

As a further measure of cytotoxic activity of JH serum, on the fourth day of incubation in the above described experiments, cells from triplicate cultures were harvested, washed five times in MEM, resuspended in MEM containing 10% pooled serum, stimulated with PHA-M (50 μl) and returned to incubation for another 3 days. The PHA responses of DH lymphocytes which had been pre-incubated with JH serum were comparable to control responses (Table 2).

TABLE 2. Lymphocyte responses to PHA following 4-day pre-incubation with JH serum

Cells*	Pre-incubation serum	No PHA	PHA
JH	Pooled	719	82364
	JH	993	99428
Husband (DH)	Pooled	324	148201
	JH	152	132970

* Cells for this experiment were recovered from cultures on the fourth day of incubation with MEM and indicated sera and cultured for an additional 3 days with MEM and pooled serum.

4. *Inhibition of PHA responses*

JH serum depressed the *in vitro* PHA responses of allogeneic lymphocytes (Table 3). In these limited studies, lymphocytes were stimulated with only one concentration of PHA-M (50 λ) which had been shown in previous experiments to give maximal stimulation to lymphocytes from healthy persons. JH serum also depressed PHA responses of autologous lymphocytes and those of UNR-2, an individual whose cells were not inhibited as stimulators in MLC reactions (Table 1). As described in the cytotoxicity experiments, the inhibitory effect of JH serum on PHA responses was completely reversible if leucocytes which had been previously incubated for 4 days with this serum were extensively washed and re-cultured in medium containing pooled serum.

5. *Titre of inhibitory activity in JH serum*

As can be seen in Fig. 1, the inhibitory activity of JH serum was demonstrable in dilutions of 1:1,000,000 and 1:100,000 in MLC with autologous responder cells and the husband's or an unrelated person's cells as stimulators. The shape of these curves suggests that the inhibitory effect is the result of specific factor(s) and not due to absence of some nutritional factor. All cultures contained a total concentration of 12·5% serum with decreasing amounts of JH serum (from 5 to 0·000005%).

TABLE 3. Effects of serum JH 2.71 on lymphocyte responses to PHA* (cpm H_3T)

Lymphocytes	Pooled serum	JH 2.71
JH	75637	19770
	120966	93949
Husband	175587	21624
	94100	21425
UNR-2	64448	14332
UNR-3	196726	27604
UNR-4	95851	8395

UNR = unrelated to JH or to her husband.

* All unstimulated cultures had responses below 800 cpm.

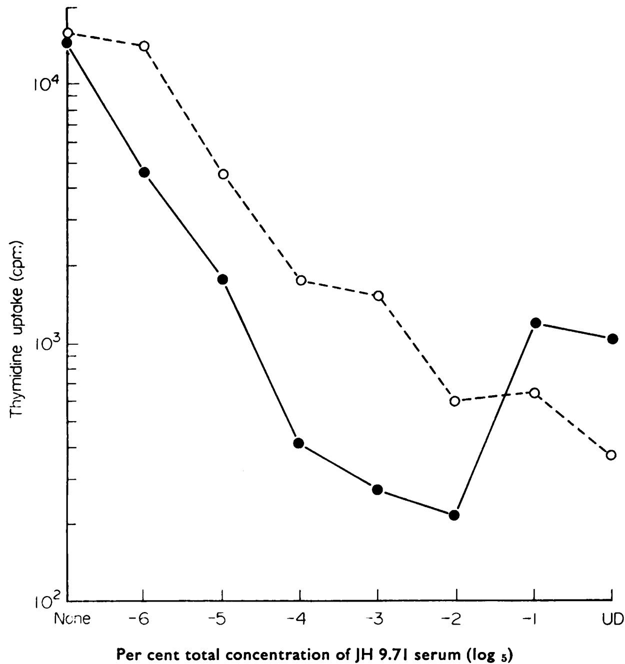

FIG. 1. Inhibitory effects of increasing dilutions of JH 9.71 serum on two MLC combinations. ●, JH × Husband$_m$; ○, JH × UNR$_m$.

6. *Absorption of serum inhibitory activity with leucocytes*

Because of the remarkably strong inhibitory activity of JH serum, initial attempts at absorption using undiluted sera gave only equivocal results. When a 1 : 100 dilution of 2 ml of JH serum was absorbed with 50-ml packed cells from DH, the inhibitory effect against an MLC of JH × DHm was no longer demonstrable. Eluates from DH cells used for absorption of undiluted JH 2.71 serum were recovered by four different methods (see Materials and Methods). All four fractions contained marked inhibitory activity.

7. *Molecular characteristics*

JH serum was precipitated with 40% saturated ammonium sulphate, resuspended, and precipitated again with 33% saturated ammonium sulphate. All fractions were dialysed for

TABLE 4. Effects of JH 2.71 serum on MLC (cpm H_3T) after saturated ammonium sulphate precipitation

Test fraction	JH × Husb$_m$	UNR-5 × Husb$_m$
Pooled serum	15836	30079
JH 2.71	7556	1168
$(NH_4)_2SO_4$ supernatant	25283	49561
$(NH_4)_2SO_4$ precipitate	2353	470
Sup + ppt (= 1:2)	664	1211
JH 2.71 (1:2)	6091	1302

3 days against 4 litres of phosphate buffered saline. Inhibitory activity was recovered in the precipitated fractions (Table 4). The first supernatant fraction was no longer inhibitory to two MLC combinations.

JH serum was filtered over a DEAE-Sephadex column using a 0.02 M phosphate buffer (pH 6.3) to isolate the 7S IgG fraction. The molarity of the buffer was then increased to 0.5 M NaCl in order to recover the remaining serum fraction. The contents of these fractions were confirmed by immunoelectrophoresis. Each fraction was concentrated back to original volume by pervaporation and then dialysed for 2 days against phosphate buffered saline and for 1 day against tissue culture medium. Inhibitory activity was strong in the IgG fraction and was absent from the serum fraction from which most of the IgG had been removed (Table 5).

TABLE 5. Effects of JH 2.71 serum fractions on MLC after filtration over DEAE-Sephadex

MLC	Test fraction*	cpm
JH × $Husband_m$	Pooled serum	10461
	JH 2.71	4088
	'IgG fraction'	788
	'IgG-depleted serum'	10556

* All cultures contained 7·5% pooled serum.

TABLE 6. Effect of adding JH 2.71 serum to cultures at varying times during incubation

Time of addition (hr)	JH × $Husband_m$		UNR-6 × UNR-7_m	
	Pooled serum	JH 2.71	Pooled serum	JH 2.71
0	16107	5425	19436	1820
3	18708	6889	19208	5233
6	16343	4457	12158	3921
9	14235	15873	19164	17731
18	15120	15722	19940	11212
24	12702	12610	18929	17122
48	15545	15741	12247	15487

8. *Kinetics of inhibition by JH serum*

No inhibitory effect was noted when either stimulator or responder cells were pre-incubated with JH serum. Cells were incubated for varying periods of time ranging from 30 min to 24 hr followed by extensive washing prior to their addition to mixed lymphocyte cultures.

The time of addition of the inhibitory serum to MLC appeared to be important (Table 6). If the serum was added during the first 6 hr of the 7-day incubation period, inhibition was seen. When the serum was added 9 hr or later after the leucocytes had been mixed together, no inhibition was noted.

The effect of JH serum on RNA synthesis of MLC cultures, terminated at 3–5 days, was evaluated by incorporation of ^{14}C-labelled uridine. In those cultures containing 5% JH

serum (and 7·5% pooled serum) incorporation of labelled uridine was markedly inhibited (i.e. 20% of controls).

In three experiments using goat antisera against human IgG or Ig light chains, abrogation of inhibition was observed with an anti-L chain antiserum but not with an anti-IgG antiserum. In the latter situation, JH serum was still inhibitory to MLC. In each experiment, the antiserum was mixed with JH serum (1 part + 3 parts, respectively) prior to addition of the mixture to the cultures. Neither the anti-L chain antiserum nor the anti-IgG antiserum was stimulatory or inhibitory to MLC responses. Further studies along these lines are being continued.

9. *Specificity of inhibition*

It seemed clear from early experiments that inhibition of MLC by JH serum was not directly related to HL-A specificities. Our experience to date includes over thirty individuals whose cells stimulate JH cells poorly in MLC when JH serum is present. In many of these MLC combinations the stimulator and responder populations have been reversed: JH sera are also inhibitory toward stimulator cells in these cultures. (Table 1, line 9). Two of the thirty persons studied have four identifiable HL-A antigens and bear no similarity to the cells of DH, the husband of JH.

As described above, JH serum inhibited MLC of JH cells against those of her children who carried different paternal haplotypes, thereby establishing either the presence of at least two separate inhibitory factors or a lack of correlation between HL-A specificities and inhibition by JH serum. In order to identify haplotypes in the H family it was necessary to combine the results of HL-A typing of family members from three generations with the results of MLC between siblings. We concluded from these studies that DH must be homozygous for HL-A 12. The presumptive haplotypes of Family H members are shown in Fig. 2. The results of MLC are given in the legend.

In order to further evaluate the possibility that the inhibitory factor(s) in JH serum might

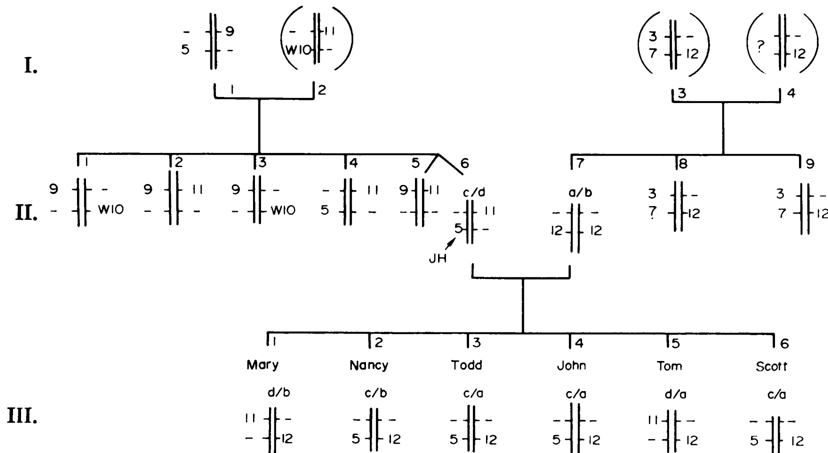

FIG. 2. Presumptive haplotypes of Family H members. MLC results: $II_8 \times II_9$, positive; $III_1 \times III_5$, positive; $III_2 \times III_4$, positive; $III_2 \times III_6$, positive; $III_4 \times III_6$, negative. Bracketed individuals were not tested. 'Mary' is the original propositus who presented with acute lymphatic leukaemia.

involve an antibody against a tissue antigen (of DH) which JH does not possess, the effect of JH serum was studied on MLC combinations involving leucocytes from siblings of JH The leucocytes of these siblings were used as stimulator or responder cells against JH cells: inhibition was noted in all combinations. A similar study of JH cells against those of the siblings of her husband also showed inhibition in all combinations. The latter experiments again were presumed to test the effects of JH serum on MLC involving each of her husband's two haplotypes (see Fig. 2).

The family of UNR-2 was studied in a similar manner in order to determine if perhaps the leucocytes of the only sibling of UNR-2 might also escape inhibition of MLC responses by JH serum. The results are shown in Table 7. JH serum inhibited the responses of JH cells against leucocytes of all family members except UNR-2.

10. *Stability of inhibitory activity with storage*

Most specimens tested after storage at −70°C for 20 months were still very active inhibitors of MLC responses. Occasional stored specimens have also been inhibitory toward UNR-2 cells.

TABLE 7. Effect of JH serum on MLC responses of family members of UNR-2

MLC	HL-A type of stimulator cells	Pooled serum	JH 11.71
JH × $father_m$	2, (FJH)	12184	1265
JH × $mother_m$	5, 10	11854	1274
JH × UNR-2_m	2, 5, 10	12156	13134
JH × $brother_m$	2, 5	15122	2688

11. *Persistence of inhibitory activity in donor*

While it has not been practical to titre the inhibitory activity of JH serum at regular intervals, 100 μl of fresh whole JH serum appeared to give approximately the same degree of inhibition in November 1971, 16 months after delivery, as was first observed 3 months after delivery of her last child.

12. *In vivo effect of JH sera*

Because a suitable sibling donor for bone marrow transplantation could not be found for the daughter of JH, an attempt was made to establish a bone marrow graft using the patient's father, DH, as the marrow donor and the mother's (JH) serum as a hopeful means of suppressing a graft-versus-host reaction. In preliminary studies on the patient, JH serum was first injected subcutaneously, followed by intravenous injection of 1 ml and later 5 ml with no change in vital signs or any untoward effects. Following total body irradiation with 950 R and bone marrow transplantation with 9×10^9 nucleated cells, the patient was given 25 ml of irradiated JH serum every other day in addition to a Storb–Thomas regimen of methotrexate immunosuppression (Storb *et al.*, 1970; Thomas *et al.*, 1971). Four weeks post-transplantation, there was no clear evidence of a graft 'take'. The patient was transplanted

again. Several days later she died with overwhelming sepsis. Post-mortem examination of bone marrow and other lymphoid organs showed no leukaemic cells.

DISCUSSION

Perhaps the most important lesson that these studies can teach us is the danger we invite when autologous serum is used in studies of *in vitro* lymphocyte responses (Gatti, 1971). In this family especially, when JH serum was part of a serum pool used for MLC studies, a sibling who was in reality a one haplotype histocompatibility mismatch might have been chosen on the basis of HL-A identity and MLC unresponsiveness as a donor for bone marrow transplantation. Most likely a fatal graft-versus-host reaction would have resulted. Using serum from AB donors would be safest; however, we have found that serum from young male donors who have no history of having received blood transfusions gives satisfactory results.

MLC responsiveness is thought to reflect antigenic differences controlled by one genetic histocompatibility region closely linked to HL-A in man (Amos & Bach, 1968; Yunis & Amos, 1971); to RhL-A in monkey (Appleman & Balner, 1972) and to H_2 in mouse (Klein *et al.*, 1973). Conversely, unresponsiveness in MLC between immunologically intact individuals is strong evidence for histocompatibility. A factor which inhibits the MLC reaction between histoincompatible persons most likely acts directly or indirectly upon the recognition sites responsible for these reactions between allogenic cells. It seems clear that JH serum possesses such a factor(s) which does not recognize any known HL-A specificity. While it is possible that the inhibitory factor is directed against another part of the HL-A region, such as an MLR locus as postulated by Yunis and Amos (1971), the haplotype studies of inhibition within Family H offer evidence against this possibility. On the other hand, if the husband (DH) were homozygous at such an MLR locus, inhibition of MLC involving children with different paternal haplotypes would not negate the above possibility.

Because of the broad specificity of this serum to inhibit responses involving more than thirty unrelated individuals while, on the other hand, consistently recognizing the cells of UNR-2, it seems likely that the JH inhibitor recognizes a more general lymphocyte receptor or product. The effect of JH serum on PHA responses, an observation also made by Ceppellini (1971) and Buckley *et al.* (1972) with similar MLC inhibitors, also suggests that the inhibition involves T-cells The recognition site may be located on cell surfaces or may be a product which is released by activated T-cells. We have not here addressed the question of whether this receptor might also be common to other cell types.

Our data indicate two distinct populations: one whose lymphocytes are inhibited by JH serum, the other not; suggesting genetically-controlled allotypes similar to the theta and IR markers of mice. It is possible to imagine that such genetic propensities might even play a role in the etiology of leukaemia. Indeed, the situation in this family is reminiscent of the theta-negative AKR mice with their high incidence of spontaneous leukaemia. The elusiveness of the recognition site of the JH inhibitor for the lymphocytes of so many unrelated individuals suggests that this site may only be expressed by transforming or differentiating lymphocytes. Perhaps it is at this stage of leukaemogenesis that immune surveillance might fail if the transformed lymphocytes were genetically incapable of expressing a particular antigen necessary for their recognition and rejection. Lymphocytotoxic antibodies against

HL-A antigens have been demonstrated in pregnant women as early as 6½ weeks of pregnancy, long before the development of any leucocytes in the embryo, thus prompting one investigator to suggest that the trophoblast, not leucocytes, may be responsible for this early stimulation (van der Werf, 1971).

While the evidence presented herein suggests that the inhibitory factor(s) contains an immunoglobulin, a strong possibility remains that an antigen–antibody complex may produce the inhibition (Sjögren *et al.*, 1971; Oppenheim, 1972). Similar MLC inhibitors have been localized in the IgG fraction by Leventhal *et al.* (1970), Hattler, Karesh & Miller (1971), Miller, Hattler & Johnson (1971), Ceppellini (1971) and Buckley *et al.* (1972). Several unsuccessful attempts have been made by one of the authors (R.A.G.) to deplete JH serum of all antibody by absorption with human anti-light chain antiserum bound to Sepharose. In each case, failure to remove the inhibitory activity was attributed to technical difficulties and these studies are continuing; however, it is also possible that the inhibitor is complexed into a matrix with antigen that may make it difficult to absorb completely with antisera.

Lastly, it is hoped that such 'natural' serum inhibitors of MLC responses will also inhibit *in vivo* responses such as graft-versus-host (GVH) reactions encountered during bone marrow transplantation. At least one clinical experience using such a serum therapeutically has been encouraging in that little or no GVH reaction was seen in an infant with severe combined immunodeficiency following a bone marrow transplant from his mother (Buckley *et al.*, 1971). In our clinical trial of JH serum, the expected GVH reaction also was not seen following transplantation of hemi-allogeneic bone marrow. On the other hand, it is entirely possible that while such sera are not demonstrably cytotoxic *in vitro*, they may be cytotoxic *in vivo*. Another possibility is that the inhibitor in JH serum may have interfered with establishment of the graft by altering cell traffic or distribution after combining with a surface receptor. Mason & Warner (1970) have inhibited GVH reactions in mice by pretreatment of donor cells with rabbit antiserum against mouse L-chain determinants. Riethmüller, Rieber & Seegas (1971) have similarly suppressed GVH reactions in mice by pretreatment of donor cells with rabbit univalent Fab-anti M (mouse) immunoglobulin. Greaves, Torrigiani & Roitt (1971) have observed inhibition of MLC responses by pretreatment of human lymphocytes with rabbit Fabγ monomer against human L-chain determinants.

Further evidence is accumulating that similar serum inhibitors of MLC responses appear in the circulation shortly after renal (Hattler *et al.*, 1971; Miller *et al.*, 1971) and ectopic heart transplantation (Heron, 1972). While their role in the success or failure of organ transplantation is not yet clear, it seems fair to assume on the basis of numerous animal and clinical studies that *in vitro* suppression of MLC responses implies *in vivo* suppression of cell-mediated immunity and graft rejection mechanisms. Such 'natural' immunosuppressants would have the additional advantages of low toxicity and relatively long survival *in vivo*.

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