

CYTOTOXICITY IN GRAFT-VERSUS-HOST REACTION

I. ROLE OF DONOR AND HOST SPLEEN CELLS

BY JAGAT N. SINGH, E. SABBADINI, AND A. H. SEHON

(From the Department of Immunology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3)

(Received for publication 3 March 1972)

The graft-*versus*-host (GVH)¹ reaction induced in F₁ hybrids by immunocompetent cells of parental origin is characterized by profound damage to the recipient, which suggests that a cytotoxic mechanism may be underlying the pathogenesis of GVH disease (1). Because of the direction of the GVH reactions, it would be expected that donor cells become sensitized against transplantation antigens of the recipient and induce specific lysis of cells carrying such antigens. However, *in vivo* studies have strongly suggested that cytopathic phenomena in GVH reaction may not be specific and that "innocent bystanders" may be affected (2, 3). Similarly, under *in vitro* conditions, spleen cells from F₁ hybrid mice undergoing GVH reactions were recently shown (4) to exert a nonspecific cytotoxic effect upon target cells of syngeneic, allogeneic, or xenogeneic genotype to the parental strain. Moreover, the cytotoxic effect was shown to be dependent on the intensity of GVH reactions and necessitated direct contact between target cells and metabolically active spleen cells; no lysis could be induced with the cell-free supernatant of cultures of these spleen cells (4).

In the present investigation, in an attempt to elucidate the nature of the "nonspecific" effect(s) in GVH reaction, the role of the donor and host cells in the pathogenesis of GVH disease was examined by using irradiated and non-irradiated hosts. From the results to be reported it was concluded that GVH reaction involved two types of cytotoxicity: (a) specific cytotoxicity due to activation of the donor cells in the process of sensitization against the target cells of the host and (b) nonspecific cytotoxicity due to host cells activated by an unknown mechanism during GVH reaction.

Materials and Methods

Animals.—Mice 8–10-wk old, of the inbred strains A/J (*H-2^a*), C57BL/6J (*H-2^b*), and C3H/HeJ (*H-2^k*), as well as F₁ hybrids, B6AF₁ (C57BL/6J × A/J), were obtained from Jackson Laboratory, Bar Harbor, Maine, while F₁ hybrids C3AF₁ (C3H/HeJ × A/J) were bred in the animal house of the Department of Immunology of the University of Manitoba, Winnipeg.

Induction of GVH Reactions.—GVH reactions were induced by intravenously injecting

¹ *Abbreviations used in this paper:* GVH, graft-*versus*-host; SaI cells, sarcoma I cells; TC 199, tissue culture medium 199.

spleen cells of parental strain origin (1×10^8 cells/recipient, unless stated otherwise prepared, as previously described in medium TC 199 [5]) into 6–8-wk old F_1 hybrids. The intensity of GVH reaction was measured with the spleen assay of Simonsen (1) and was expressed in terms of the “spleen index.”

Target Cells.—The following cell types were used as targets for the in vitro study of cytotoxicity. Sarcoma I (SaI) cells were grown as ascites tumors in mice of strain A/J. B16 melanoma cells from a solid tumor transplantable in C57BL/6J mice were grown in primary monolayer cultures before use as target cells. SaI cells were harvested 4–6 days after intraperitoneal injection and were treated with a hypotonic solution (5) to lyse any red blood cells. The monolayer cultures were brought into suspension by incubation for 10–20 min at 37°C with 0.25% trypsin in Madin-Darby's solution (6). The cells were then washed with TC 199 and labeled with ^{51}Cr as previously described (5).

Specific Sensitization of C57BL/6J Mice.—C57BL/6J mice were sensitized against the transplantation antigens of A/J strain with skin grafts. 2 wk after skin grafting, a booster injection of 1×10^7 SaI cells per mouse was given by the intraperitoneal route. 7 days after injection of SaI cells, spleen cells of these animals were used for specific lysis of SaI target cells.

Lysis of B6AF₁ Hybrid Cells with Alloantiserum and Complement.—To lyse B6AF₁, spleen cells an alloantiserum raised in C57BL/6J mice treated with SaI cells (7) was used. This serum lysed normal A/J and B6AF₁ spleen and lymph node cells (50% lysis in 1:1200 dilution), but had no effect on C57BL/6J lymphocytes. Portions of 5×10^6 spleen cells (maximum) were mixed with the alloantiserum (final dilution in TC 199 was 1:200) in a total volume of 2.0 ml. After incubation for 30 min at 37°C , the cells were separated by centrifugation and were then resuspended in 2.0 ml. of fresh guinea pig serum as a source of complement (previously absorbed with B6AF₁ spleen cells at 4°C) at a final dilution of 1:10. The cell suspensions were further incubated for 30 min at 37°C and were washed once, before using them in experiments for target cell lysis. The viability of the spleen cells was also checked by trypan blue dye exclusion test.

Irradiation.—Mice were subjected to whole body gamma irradiation using a ^{60}Co source. The source to mid-body line distance was 100 cm; the dose rate was 80 rads/min.

Assays for Target Cell Lysis.—To demonstrate the cytotoxic effects of spleen cells from mice undergoing GVH reactions, two methods were adopted: (a) In vitro cytotoxicity of lymphoid cells were ascertained in terms of the release of ^{51}Cr from the target cells (4). (b) After in vitro incubation of spleen cells with the target (tumor) cells, the in vivo growth of tumor in syngeneic recipients was studied by an adaptation of the method described by Winn (8).

The in vitro cytotoxic assay, previously described in detail (5), consisted of mixing the spleen cells of mice undergoing GVH reaction (1×10^7 cells) with ^{51}Cr -labeled target cells ($1-2 \times 10^5$ cells) in 1-ml volumes of TC 199 in plastic test tubes. This assay was also used on a microscale in microtissue culture plates with flat-bottom wells (Falcon Plastics, Division B-D Laboratories, Inc., Los Angeles, Calif.). Each well contained 1×10^6 spleen cells in 0.2 ml volume and 2×10^4 target cells in 0.05 ml volume. Thus the final volume per well was 0.25 ml. The plates were sealed and maintained at 37°C for 16 hr. After the incubation, the plates were spun at 250 g for 10 min and 0.1 ml volumes of the cell-free supernatants were collected for determination of the ^{51}Cr released. The two procedures will be referred to as macro- and microcytotoxicity tests. The corrected per cent lysis was calculated by the procedure described before (4).

RESULTS

Nonspecific Cytotoxicity due to GVH Reactions.—The results obtained with spleen cells from C3AF₁ hybrid mice, 8 days after injection of spleen cells of syngeneic or parental strains, are illustrated in Fig. 1. As is evident, significant

cytotoxic effects of spleen cells of C3AF₁ mice, undergoing GVH reaction, on target cells of related and unrelated genotypes to the parental cells (i.e. SaI and B16, respectively), were obtained. These results are confirmed by the data listed in Table I, which demonstrate the nonspecific cytotoxic effect observed in a different parent → F₁ hybrid combination (A/J → B6AF₁). Moreover, the cytotoxicity effect was directly related to the GVH reaction (measured in terms of the spleen index) as shown by the absence of both cytotoxicity and splenomegaly when syngeneic (B6AF₁) or sonicated parental (A/J) cells were injected into F₁ hybrids.

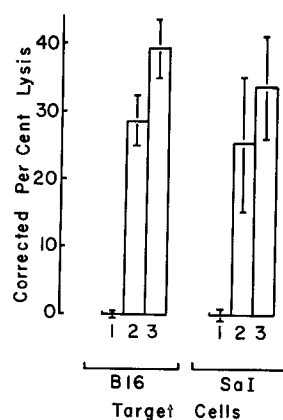


FIG. 1. Lysis of target cells incubated with spleen cells of F₁ hybrid mice which had received 1×10^8 spleen cells from syngeneic or parental strains 8 days earlier. 1. C3AF₁ → C3AF₁; 2. C3H/HeJ → C3AF₁; 3. A/J → C3AF₁.

Determination of Nonspecific Cytotoxic Response due to GVH Reaction at Various Time Intervals after the Induction of GVH Reactions.—In order to study the cytotoxic effect of spleen cells from B6AF₁ hybrid mice at various time intervals up to 64 days after the induction of GVH reactions, B6AF₁ hybrid mice were injected with parental (A/J) spleen cells (1×10^8 cells per mouse). The control groups received the same number of syngeneic B6AF₁ spleen cells per mouse. Cytotoxic effects of spleen cells from mice undergoing GVH reaction were studied with SaI cells as targets. As can be seen from the results shown in Fig. 2, there was a slight cytotoxic response on day 4 after the induction of GVH reaction, which was followed by a marked increase in cytotoxicity up to day 8 and then a gradual decrease in the cytotoxic potential of the spleen cells.

Specific Lysis of F₁ Hybrid Cells with Alloantiserum.—To study the respective roles of host and donor cells in the induction of nonspecific cytotoxic effects, the spleen cells of B6AF₁ mice undergoing GVH reaction induced with C57BL/6J spleen cells (8 days after intravenous injection of 1×10^8 cells per mouse) were

treated with a C57BL/6J anti-A/J serum. This treatment resulted in lysis of the F₁ hybrid host cells but had no effect on the viability of the C57BL/6J donor cells. After this treatment, cells were washed once and then mixed with ⁵¹Cr-labeled B16 melanoma target cells for microcytotoxicity test. As shown in Table II, pretreatment of B6AF₁ hybrid spleen cells undergoing GVH reaction with alloantiserum and complement completely abolished their cytotoxic activity. Antiserum or complement alone had no cytotoxic effect on the B6AF₁ hybrid

TABLE I
Spleen Index and Corrected Per Cent Lysis of SaI Target Cells with Spleen Cells of B6AF₁ Hybrid Mice Which Had Received 1×10^8 Spleen Cells from Syngeneic and Parental (Both Untreated or Sonicated) Cells 8 Days Earlier

Treatment Donor → Recipient	No. of animals per group	Spleen index	Corrected per cent lysis
		Mean ± SE	Mean ± SE
B6AF ₁ → B6AF ₁	3	1.00 ± 0.07	0.00 ± 2.71
A/J → B6AF ₁	3	3.67 ± 0.14	45.48 ± 6.21
A/J (sonicated) → B6AF ₁	3	0.91 ± 0.07	4.43 ± 2.42

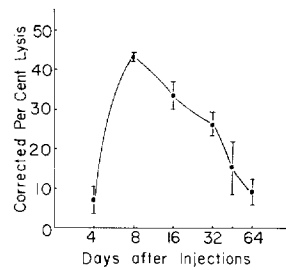


FIG. 2. Cytolytic effect of spleen cells from mice undergoing GVH reaction as a function of time after injection of parental spleen cells into F₁ hybrid mice. GVH reactions were induced by injecting A/J spleen cells into B6AF₁ (1×10^8 /mouse). SaI cells were used as targets.

cells, as judged by trypan blue dye exclusion test and by their inability to affect the cytotoxic potential of the spleen cells from mice undergoing GVH reaction. These results clearly suggest that F₁ hybrid cells were responsible for the observed nonspecific cytotoxicity. The possibility that C57BL/6J cells might have been indirectly affected by the lysis of B6AF₁ cells was excluded in an experiment in which specifically sensitized C57BL/6J cells (from mice grafted with A/J skin) were mixed with B6AF₁ spleen cells in different proportions. The sensitized C57BL/6J cells still induced lysis of SaI cells under these conditions and their activity was not affected by treatment with C57BL/6J anti-A/J serum in presence of complement (Table III).

Nature of Cytotoxicity due to GVH Reactions in Irradiated and Nonirradiated

Mice.—To investigate further the role of host cells in the induction of non-specific cytotoxic effects, B6AF₁ hybrid mice were irradiated and treated with spleen cells of parental origins. 6 days after the injections, spleen cells from the recipient mice were incubated with labeled target cells of both parental genotypes. The results of an experiment, in which a lethal dose of radiation (900

TABLE II
Effect of C57BL/6J Anti A/J Alloantiserum on the Lysis of B16 Melanoma Target Cells Incubated with Spleen Cells of F₁ Hybrid Mice Which Had Received Spleen Cells from Syngeneic or Parental Cells 8 Days Earlier

Injections		Corrected per cent lysis*	
Donor → Recipient	No. of animals per group	In vitro treatment of lymphoid cells	
		No treatment	Spleen cells treated with antiserum + complement
<i>Mean ± SE</i>			
B6AF ₁ → B6AF ₁	3	0.00 ± 0.81	-3.53 ± 1.28
C57BL/6J → B6AF ₁	4	16.02 ± 2.53	-3.83 ± 1.38

* The relatively low percentage of lysis obtained in these experiments was due to the fact that a small number of spleen and target cells were used to reduce the amount of antiserum necessary for complete lysis of F₁ hybrid cells.

TABLE III
*Effect of a C57BL/6J Anti-A/J Alloantiserum on the Lysis of SaI Target Cells Incubated with Spleen Cells from C57BL/6J Mice Sensitized to A/J Antigens**

Treatment	No. and source of spleen cells × 10 ⁴ /well.			Corrected percent lysis
	Sensitized C57BL/6J	+	Untreated B6AF ₁	
<i>Mean ± SE</i>				
No treatment	50	+	50	49.66 ± 5.7
	25	+	75	41.75 ± 2.7
	10	+	90	14.33 ± 4.5
Antiserum + complement	50	+	50	43.50 ± 3.1
	25	+	75	37.36 ± 2.5
	10	+	90	10.69 ± 6.8

* Cytotoxicity was observed with or without the lysis of B6AF₁ hybrid cells added to the sensitized C57BL/6J spleen cells.

rads) was used, are illustrated in Table IV. Spleen cells from the irradiated recipients undergoing GVH reaction exerted a specific cytotoxic effect on the target cells carrying the antigens against which the donor cells had been sensitized in the F₁ hybrid hosts. Spleen cells from nonirradiated recipients, also undergoing GVH reaction, exerted a nonspecific cytotoxic effect, i.e., both parental target cells tested were equally affected. Furthermore, there was no splenomegaly observed in the irradiated recipients, thereby suggesting that the

response of host cells had been completely suppressed due to lethal irradiation. On the other hand, quite pronounced splenomegaly was observed in the non-irradiated recipients. These results also point to the fact that the host cells are indeed the effectors of nonspecific cytotoxicity in GVH reaction. The dose of irradiation was critical since the spleen cells of F_1 hybrid mice treated with sublethal doses (300–700 rads) and injected with parental spleen cells induced nonspecific cytotoxic effects of a similar order of magnitude as nonirradiated hosts, while the dose of 900 rads consistently suppressed the nonspecific cytotoxicity.

Growth of SaI Cells in Syngeneic Hosts after Incubation with Spleen Cells from

TABLE IV

Lysis of Target Cells Incubated with Pooled Spleen Cells of F_1 Hybrid Mice Which Had Received Spleen Cells from Syngeneic or Parental Cells 6 Days Earlier

Treatment Donor → Recipient	Corrected percent lysis	
	Target cells used	
	Sarcoma I ($H-2^a$)	B16 melanoma ($H-2^b$)
	Mean ± SE	
Irradiated recipients*		
B6AF ₁ → B6AF ₁	0.00 ± 2.5	0.00 ± 1.3
C57BL/6J → B6AF ₁	36.28 ± 3.6‡	0.01 ± 1.1
A/J → B6AF ₁	3.97 ± 1.0	33.58 ± 2.9‡
Nonirradiated recipients		
B6AF ₁ → B6AF ₁	0.00 ± 3.7	0.00 ± 2.6
C57BL/6J → B6AF ₁	55.70 ± 4.2‡	46.04 ± 5.1‡
A/J → B6AF ₁	47.80 ± 7.6‡	43.23 ± 6.3‡

* 900 rads whole body irradiation.

‡ $P < 0.01$.

Mice Undergoing GVH Reaction.—To complement the results of the in vitro assay and to establish if lysis of target cells was indeed induced by spleen cells of mice undergoing GVH reaction, the capacity of tumor cells to grow in syngeneic recipients was studied. For this purpose SaI cells were treated in the following ways:

Experimental group 1: SaI cells (5.0×10^6) were incubated in a tissue culture flask on a rocking platform for 16 hr at 37°C in TC 199 (25 ml) supplemented with fetal calf serum and antibiotics in the absence of lymphoid cells before in vivo inoculation.

Experimental group 2: SaI cells (2.5×10^6 in 25 ml of TC 199 supplemented with fetal calf serum and antibiotics) were incubated for 16 hr at 37°C in the presence of 2.5×10^8 spleen cells from B6AF₁ mice which had received 1×10^8 syngeneic spleen cells 8 days earlier by intravenous route.

Experimental group 3: The same numbers of SaI and spleen cells from mice undergoing GVH reaction per flask were incubated under the similar conditions

as described for experimental group 2. The GVH reactions had been induced by injection of 1×10^8 parental (A/J) spleen cells into B6AF₁ hybrid mice, 8 days earlier by intravenous route.

After incubation, the cells from these three experimental groups were collected in sterile plastic tubes, centrifuged (250 g for 10 min), resuspended in TC 199 to a concentration 5×10^6 SaI cells/ml, and 0.1-ml volumes of this suspension was injected subcutaneously into A/J mice. Each experimental group consisted of eight mice and, 7 days after inoculations, the tumors were removed and weighed. The results of this experiment, reported in Fig. 3, demonstrate that spleen cells of mice undergoing GVH reaction markedly reduced tumor growth (group 3) in

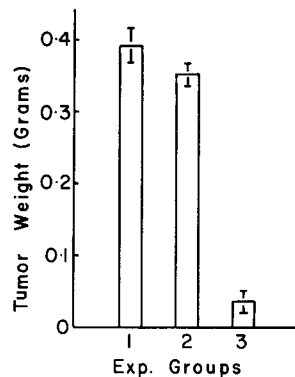


FIG. 3. Growth of SaI cells in A/J mice after *in vitro* incubation of these cells for 16 hr under different conditions. 1. SaI cells alone; 2. SaI cells + control spleen cells; 3. SaI cells + GVH reaction spleen cells.

comparison with the controls, thus confirming the cytotoxic nature of the reactions previously studied with the ^{51}Cr technique. It is also clear from these results that spleen cells of the control groups did not exert any significant cytotoxic effect on the tumor cells, since the mean growths of SaI cells incubated without or with lymphoid cells, i.e. groups 1 or 2, respectively, were not markedly different.

DISCUSSION

The purpose of this study was to gain some insight into the mechanism by which lesions of GVH disease are induced and to clarify the respective roles of donor and recipient cells in the events leading to tissue damage during GVH reaction.

To achieve the aims of this study, two methods were adopted involving *in vitro* measurements of target cell lysis, i.e. release of ^{51}Cr , and *in vivo* growth of SaI cells in syngeneic recipients. The results of both procedures showed that the

spleen cells from F₁ hybrid mice undergoing GVH reaction exerted a nonspecific cytotoxic effect upon the target cells.

The mechanism by which the spleen cells from mice undergoing GVH reaction kill the target cells of different genotypes is not clear. The findings that freezing and thawing of spleen cells, or heating them at 48°C for 30 min, abolished their cytotoxic activity indicate that living lymphoid cells were required. Furthermore, the inhibitory effect of metabolic inhibitors (2,4-dinitrophenyl and iodoacetate) on the cytotoxic potential of spleen cells (4) shows that metabolic activity is also required for the cytolytic functions.

Ramseier and Lindenmann (9) reported that F₁ hybrid animals can produce antibodies against the structural components (recognition structures) present on the parental cells reacting against antigens of the F₁ hybrids and absent in the F₁ hybrid animals. The participation of such an immune response in the present investigation is unlikely, firstly, because the target cells used in this study were not immunologically competent and, therefore, would not be expected to carry the specific recognition structures, and, secondly, because the spleen cells from nonirradiated F₁ hybrid mice undergoing GVH reaction were equally cytotoxic to target cells of both parental origins (Table IV). The fact that there exists a correlation between nonspecific cytotoxic activity and the spleen index, supports the concept that host cells are activated into a state of nonspecific cytotoxic potential as a consequence of the immunological attack by donor cells. The nonspecific cytotoxic activity of spleen cells of mice undergoing GVH reaction was maximal at day 8 after induction of GVH reaction and declined gradually with time Fig. 2. This correlates well with the known decline of immunological activity of donor cells which may be due to either (*a*) acquisition of tolerance of the donor cells to host cell antigens as suggested by Dineen (10), or (*b*) elimination of donor cells from the F₁ hybrid hosts by day 8 or later (11).

It may be suggested that during the GVH reaction, the interaction of parental cells with F₁ hybrid cells results in the release of some humoral factor(s) capable of stimulating nonspecifically the host lymphoid cells into a cytotoxic state. Factors of this nature have been described in mixed leukocyte cultures in vitro (12, 13). However, no evidence has been so far obtained to suggest the existence of similar factors in GVH reactions.

Irrespective of the mechanism responsible for its induction, the nonspecific cytotoxic activity of host cells, described here, may help to clarify some problems in the pathogenesis of GVH disease. The regressive phenomena occurring during GVH reaction, such as atrophy of lymphoid organs, cachexia, and death of the recipients have been attributed by most authors to a direct attack of donor cells on host tissues. While such an attack undoubtedly occurs, it is very difficult to explain why in most of the cases studied the attacking donor cells disappeared early after induction of GVH reaction but with no improvement in the condition of the host (11). Moreover, destruction of donor cells 6 days after

induction of GVH reaction with alloantisera (14) did not result in any improvement of the host's conditions. Therefore, some other mechanism underlying the continuation of the noxious effects, even after disappearance of the immune donor cells, may be involved. It may be suggested that host cells are activated into a state of nonspecific cytotoxicity and thus become capable of damaging other host cells.

SUMMARY

Under in vitro conditions spleen cells from nonirradiated F₁ hybrids, in which a (graft-vs.-host) (GVH) reaction had been induced with lymphoid cells of parental origin, lysed nonspecifically target cells, i.e., cells syngeneic or allogeneic to the parental genotypes. Furthermore, tumor cells exposed in vitro to spleen cells of F₁ hybrid mice undergoing GVH reaction had markedly decreased ability to grow in syngeneic recipients. Experiments involving inhibition of cytotoxicity with alloantisera indicated that this nonspecific effect was due to host cells. By contrast, spleen cells of lethally irradiated F₁ hybrids undergoing GVH reaction lysed specifically the target cells of the genotype against which the parental (donor) cells had been sensitized; this finding further supports the contribution of host cells to the nonspecific cytotoxic effects in GVH reaction.

From these results it was deduced that the cytotoxic effects during GVH reaction involve at least two processes: (a) sensitization of the donor cells to the antigens of the recipient resulting in the activation of their potential to lyse specifically the recipient's cells, and (b) activation of the host's cells into a state of nonspecific cytotoxicity, as a consequence of the immunologically specific attack of the donor cells.

The skillful technical assistance of Mrs. Elizabeth McGuire is gratefully acknowledged. This study has been supported by grants from the Medical Research Council of Canada.

BIBLIOGRAPHY

1. Simonsen, M. 1962. Graft-versus-host reactions. Their natural history and applicability as tools of research. *Prog. Allergy* **6**:349.
2. Streilein, J. W., and R. E. Billingham. 1970. An analysis of graft-versus-host disease in Syrian hamsters. II. The epidermolytic syndrome: studies on its pathogenesis. *J. Exp. Med.* **132**:181.
3. Elkins, W. L., and R. D. Guttman. 1968. Pathogenesis of a local graft versus host reaction. Immunogenicity of circulating host leukocytes. *Science (Wash. D.C.)*. **159**:1250.
4. Singh, J. N., E. Sabbadini, and A. H. Sehon. 1971. Non-specific cytotoxicity in graft-versus-host reactions. *Transplantation*. **11**:475.
5. Sabbadini, E. 1970. Studies on the mechanism of target cell lysis induced by immune cells. *J. Reticuloendothel. Soc.* **7**:551.
6. Madin, S. H., and N. B. Darby. 1958. Established kidney cell lines of normal adult bovine and ovine origin. *Proc. Soc. Exp. Biol. Med.* **98**:574.
7. Sin, Y., E. Sabbadini, and A. H. Sehon. 1971. In vitro lysis of tumor cells by

- guinea pig lymphoid cells sensitized to heterologous gamma globulins. *Cell. Immunol.* **2**:239.
8. Winn, H. J. 1959. The immune response and the homograft reaction. *Natl. Cancer Inst. Monogr.* **2**.
 9. Ramseier, H., and J. Lindenmann. 1969. F₁ hybrid animals: reactivity against recognition structures of parental strain lymphoid cells. *Pathol. Microbiol.* **34**:379.
 10. Dineen, J. K. 1961. Acquisition of graft versus host tolerance. *Nature (Lond.)*. **189**:680.
 11. Fox, M. 1962. Cytological estimation of proliferating donor cells during graft-versus-host disease in F₁ hybrid mice injected with parental spleen cells. *Immunology.* **5**:489.
 12. Kasakura, S., and L. Lowenstein. 1965. A factor stimulating DNA synthesis derived from the medium of leucocyte cultures. *Nature (Lond.)*. **208**:795.
 13. Janis, M., and F. H. Bach. 1970. Potentiation of *in vitro* lymphocyte reactivity. *Nature (Lond.)*. **225**:238.
 14. Russell, P. S. 1962. Modification of runt disease in mice by various means. *Transplant., Ciba Found. Symp.* 350.