IMMUNOLOGIC INCOMPETENCE OF IMMUNOLOGICALLY RUNTED ANIMALS*, ‡

By R. MICHAEL BLAESE,§ CARLOS MARTINEZ,|| M.D., AND ROBERT A. GOOD,¶ M.D.

(From the Pediatric Research Laboratories of the Variety Club Heart Hospital, and the Department of Physiology, University of Minnesota, Minneapolis)

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It is well established that the syndrome known as "runt disease," "wasting disease," or "homologous disease" is the result of an immune reaction elicited by the introduction of immunologically competent lymphoid cells into homologous individuals incapable of rejecting the donor cells. This graft *versus* host reaction has been induced in fetal (1), newborn (2), and adult animals previously made tolerant of the homologous cells (3) or in adults pretreated with a lethal dose of whole body x-irradiation (4). Similarly, lymphoid cells derived from one of the parent strains injected into genetically tolerant F_1 hybrids have also provoked this syndrome (5).

Vrubel (6) has reported an increase in survival time of skin homografts in rabbits exhibiting the graft *versus* host reaction as a consequence of replacing the regional lymph nodes of the host with nodes taken from donors presensitized against the recipient of the graft. This observation, together with the evidence of extreme atrophy of lymphoid tissue observed in "runts" and increased susceptibility of these animals to infection, led us to suspect that "runted" animals might be immunologically deficient. While our studies to test this hypothesis were in progress (7), Howard and Woodruff (8), using methods somewhat different from those used here, presented evidence that the graft *versus* host reaction compromises to some degree the ability of mice to form antibodies against bacterial antigens.

It is the purpose of this report to present our observations on the effect of graft *versus* host reactions on the immunologic capacity of the recipient animals

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|| American Cancer Society Research Professor of Physiology.

¶ American Legion Memorial Heart Research Professor of Pediatrics.

against bacteriophage antigens. It will be shown that injection of immunologically competent cells into immunologically tolerant animals or genetically tolerant F_1 recipients interferes with the capacity of the cells of the recipient to form antibody to T_2 bacteriophage. It will be shown further that the *donor* cells in animals undergoing graft *versus* host reaction are also rendered immunologically inadequate. Finally, data will be presented indicating that when the donor cells had received immunologic stimulation before being injected into F_1 hosts, immune responses occurred, and an anamnestic immunologic reaction was demonstrable.

Materials and Methods

Experimental Animals: Mice.—Animals of the A and C_3H/Bi strains, and F_1 hybrids resulting from the cross of A strain females and $C_{57}Bl/1$ males (AB_1F_1) were used in these studies. The mice were originally obtained from the colonies of Dr. J. J. Bittner, and have been maintained in our own colonies by rigorous inbreeding procedures since 1956. The mice were housed in plastic cages (five or fewer per cage) and offered Purina lab chow and water *ad libitum*. Each mouse was weighed three times weekly on a Mettler balance.

Cell Suspensions and Injection.—Suspensions of spleen cells were prepared by methods previously described (9). In adult animals the suspension was promptly injected into the tail vein following ether anesthesia. In the newborn C_3H animals, the viable A strain spleen cells were injected into the anterior facial vein.

Skin Homografting.—C_3H mice, injected at birth with A strain spleen cells, were grafted 40 days later with a full thickness abdominal skin graft taken from adult donors of the A strain. The grafting procedure is that used as routine in this laboratory (9). Mice retaining the grafts for at least 3 months were considered tolerant and used in the runting experiments.

Runting Models.—In the first experiment, adult $(A \times C_{57}Bl)F_1$ hybrids were given 116 to 170 million viable A strain spleen cells. In the second model, C_3H animals tolerant of A strain skin were given 130 to 170 million viable A spleen cells. Control groups included F_1 hybrids injected with isologous spleen cells, as well as untreated hybrid and A strain animals.

Antigen and Antibody Determination.—The coliphage T_2 was used as the antigen in all experiments. The phage, originally supplied by Dr. E. S. Lennox of New York University, was suspended in sterile saline, 2×10^{10} viable particles per ml as determined by plaque count. Each animal was given 0.5 ml (10^{10} particles) intraperitoneally before, at the time of, or at various intervals after administration of the cell suspensions. Blood was collected from the retroorbital venous plexus into heparinized capillary tubes 4 or more days after administration of the antigen as indicated in the figures and tables. The plasma was separated by centrifugation, and the samples stored individually at −20°C until studied.

When the antibody assays were to be made, the plasma was diluted 1:50 in a 20 per cent normal rabbit serum in saline diluent, heated at 56°C for 30 minutes, and incubated with 10^5 particles of bacteriophage per ml in the reaction tubes. The bacteriophage particles remaining active after 30 minutes, 1, 2, 4, 8, and 24 hours of incubation were assayed by the methods of Adams (10).

The antibody determinations were all made without knowledge of whether the animals had experienced runting or not.

RESULTS

Antibody Production of F_1 Hybrid Mice Runted with Parent Strain Spleen Cells.—$(A \times C_{57}Bl/1)F_1$ mice, 3 to 4 months of age, were subjected to intra-

venous injection of 130 to 170 million nucleated cells from the spleens of adult A strain donors. Control groups included F_1 hybrids injected with comparable numbers of isologous spleen cells, as well as normal uninjected (A × C_{57}Bl/1)F_1 and A strain animals.

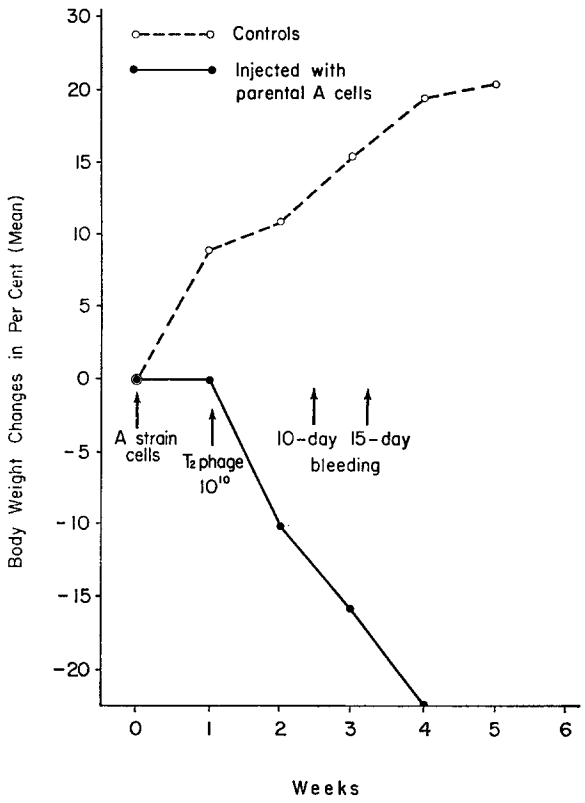

FIG. 1. Weight curve of control (A × C_{57}Bl/1)F_1 mice compared with that of immunologically runted hybrid recipients of A strain cells (at time 0) and phage (8 days after administration of cells).

About 90 per cent of the animals in the experimental group developed runt disease characterized by marked growth failure, weight loss, diarrhea, roughing of the fur, and early death. Fig. 1 outlines the plan of the experiment and presents a comparison of the weights of normal control (A × C_{57}Bl/1)F_1 hybrid mice with the weights of (A × C_{57}Bl/1)F_1 mice injected with A strain cells.

The data on the response to the intraperitoneal injection of T_2 bacteriophage were distributed according to the status of the animal: runted following injection of the parent strain cells, not runted following injection of the parent strain cells (discarded from the experiment), and controls receiving either isologous

cells or none at all. Figs. 2 and 3 show phage neutralization curves of a group of runted mice receiving bacteriophage 8 days after cell injection. Fig. 2 illustrates the results in 10-day plasma samples. The lines representing the antibody

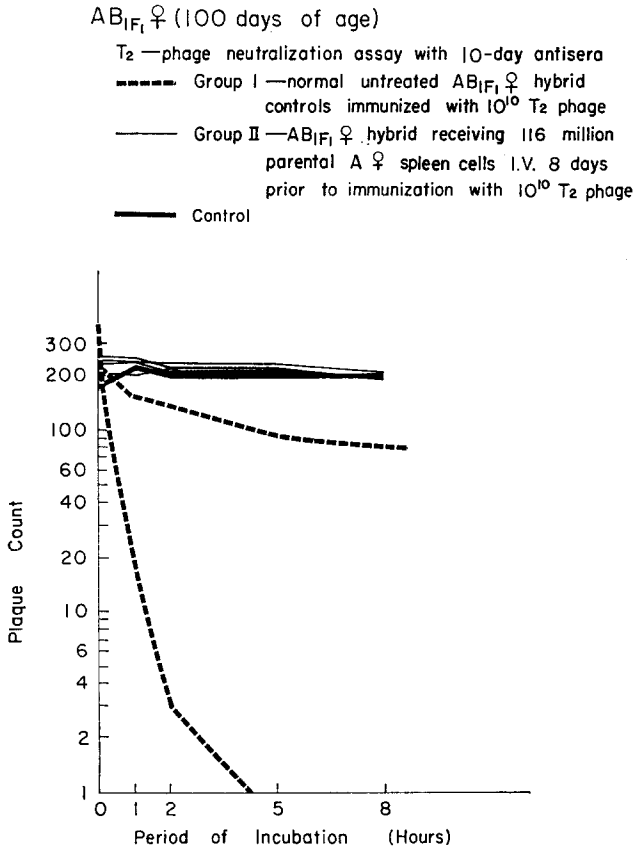

FIG. 2. Ten-day antibody response of runted AB_1F_1 (A × C_{57}Bl/1)F_1 mice to bacteriophage administered 8 days after cell injection (group II), compared to that of control hybrid animals (group I) and the diluent control. Each runted animal is represented by one of the thin lines, grouped around the diluent control. The heavy broken lines indicate the extremes of the distribution of group I, the controls. Plasma from runted animals showed no significant phage neutralizing activity over an 8 hour period of incubation.

production of individual runted mice are grouped around the line representing the diluent control, indicating that these animals developed no neutralizing antibody after injection of the bacteriophage. The uninjected control group (represented on the graph by the antibody levels of the animals at the extremes of the control distribution) all showed significant antibody production. An even greater difference is observed in Fig. 3, illustrating the results of phage assay in 15-day samples.

Antibody Production of Tolerant Mice Runted with Additional Adult Spleen Cells.—A further study of the effect of the graft *versus* host reaction on the immunologic capacity of mice was performed using C_3H mice made tolerant of

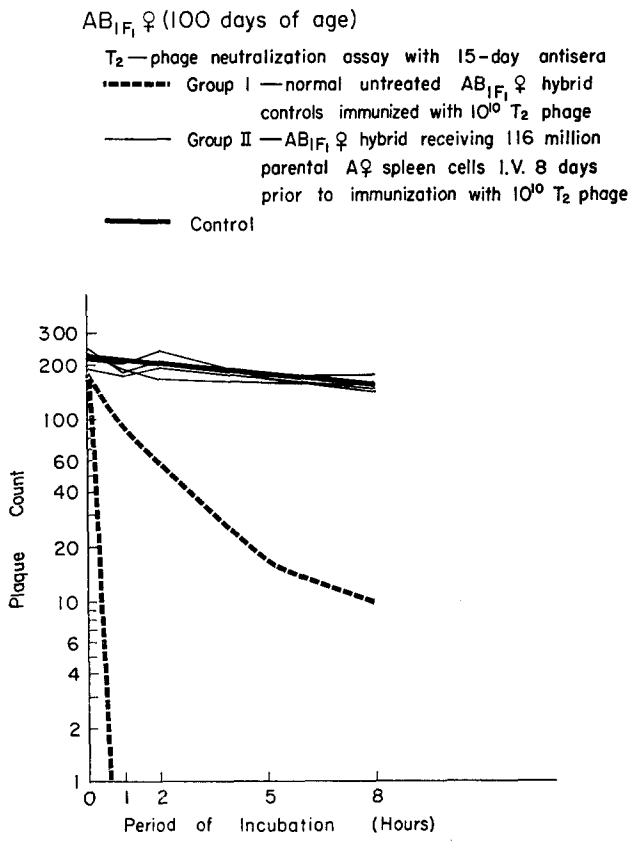

FIG. 3. Fifteen-day antibody response of runted AB_1F_1 ($A \times C_{57}Bl/1)F_1$ mice to bacteriophage administered 8 days after cell injection (group II), compared to that of control hybrid animals (group I) and the diluent control. Again, there was no significant phage neutralization by plasma from the runted animals, grouped around the diluent control. On the other hand, the phage-neutralizing activity of samples from the control animals was increased over that observed at 10 days (Fig. 2).

A strain tissue by intravenous injection of A strain spleen cells at birth. At 5 to 6 months, these mice were again injected intravenously with adult A strain spleen cells. Eight days later, the animals were challenged with 10^{10} particles of T_2 phage intraperitoneally. Phage neutralization activity was determined on samples taken 10 days following injection of the bacteriophage. When results were compiled, samples from animals that did not develop runt disease were

discarded; the remaining data are summarized in Table I and show that this second type of runted animal also had an extreme immunologic deficiency.

Effect of Time of Antigenic Challenge in Relation to Spleen Cell Injection.— In this series of experiments, T_2 phage was administered to the F_1 hybrid mice before, at the time of, and at varying intervals after the injection of the parent strain spleen cells. Phage-neutralizing activity was determined on samples taken 10 days following the antigenic challenge. As in the previous experiment, phage neutralization results from animals that did not develop runt disease were discarded.

As shown in Fig. 4, the F_1 hybrid mice challenged with antigen either 4 or 6 days before injection of the spleen cells had antibody levels equal to those of untreated controls, but those challenged with antigen 2 days before cell injec-

TABLE I

Antibody Production by C_3H Mice Tolerant of A Skin Undergoing the Graft Versus Host Reaction from an Intravenous Injection of 130 to 170 Million A Strain Spleen Cells 8 Days Prior to Antigenic Challenge

Group	Surviving plaques after 2 hrs. incubation of plasma from 10-day bleeding
	per cent
Normal C_3H	4 (2)*
Runting C_3H tolerant of A	95 (5)

* Number of animals indicated in parenthesis.

tion had significantly reduced antibody levels. When cells and antigen were given on the same day, the mice produced small but significant amounts of antibody to phage. However, all animals receiving cells before antigen produced no significant antibody during observation periods up to 38 days.

In this series, then, complete suppression of antibody production was seen consistently in runted mice, provided the antigen was administered after the injection of the adult spleen cells. On the other hand, antibody was detected in all animals stimulated with phage before or at the time of spleen cell injection. The prior antigen administration had no effect on the development of the runt disease.

Phage Neutralization by Late Serum Samples from Runted F_1 Hybrid Mice.— Runted F_1 hybrids were followed until death, with bleedings at various intervals. Table II summarizes the mean phage survival values in neutralization tests of plasma samples taken at intervals from both the runted mice and the F_1 hybrid controls. Most of the runted mice died within 50 days after administration of the parent strain cells; however, in one instance a serum sample was obtained as late as 96 days after administration of antigen. None of the runted

animals whose antigenic challenge occurred following parent strain spleen cell injection showed evidence of significant antibody production at any time. Of interest, and worthy of further study, is the observation that two mice, challenged with the T_2 antigen prior to injection of the cells, showed antibody in 10-day bleedings but showed none in 31- to 40-day samples. Control animals consistently showed more antibody in 31- to 40-day specimens than in those obtained at 10 days.

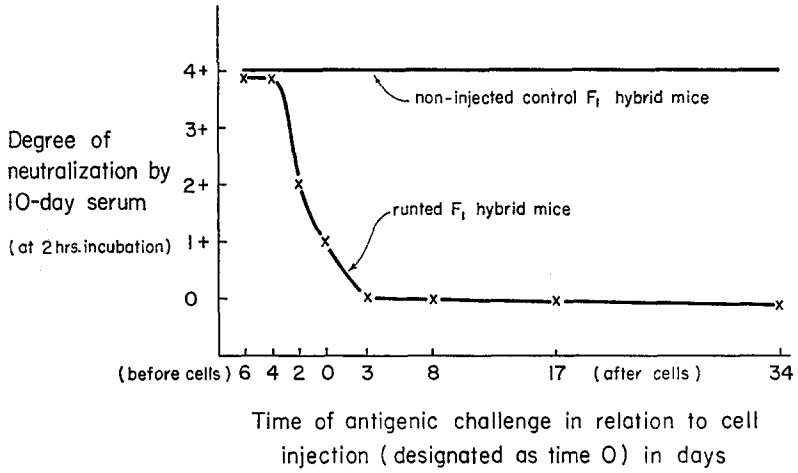

FIG. 4. Ten-day antibody response of runted (A × C_{57}Bl/1)F_1 mice to bacteriophage administered before, at the time of, and following administration of the A strain cells. When phage was given 4 or 6 days before injection of cells, the antibody response was at control levels, but when antigenic stimulation preceded cell injection by 2 days, antibody production was reduced. The failure of antibody response to bacteriophage was complete in all mice receiving antigenic stimulation after injection of the parent strain cells.

Comparison of the Antibody-Producing Capacity of A and (A × C_{57}Bl/1)F_1 Mice.—Since experimental studies (3, 11, 12) suggest that transplanted spleen cells may comprise a significant proportion of the lymphoreticular cells in the chimeric state, it seemed important to compare the antibody-producing capacity of the A parent strain and the (A × C_{57}Bl/1)F_1 hybrid. Normal 3-month-old animals of both strains were given 10^{10} bacteriophage particles. As in the preceding studies, 10 day samples were assayed for phage-neutralizing antibody. Table III summarizes the results of this study and indicates that these strains did not differ significantly in immunologic response to this antigen.

Antibody Production in (A × C_{57}Bl/1)F_1 Mice Runted with Cells from A Donors Previously Immunized with T_2 Bacteriophage.—In a further analysis of the immunologic status of the runted mice, an experiment was planned to de-

TABLE II

Antibody Production in F_1 Hybrid Recipients Runted with Parent Strain Spleen Cells

Group	Time of antigenic challenge*	Plaques surviving after 2 hrs. incubation of serum with T2 phage‡						
		6 days§	10–11 days	14–17 days	23–25 days	31–40 days	48 days	96 days
	days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Uninjected	—		24 (4)					
Runted	+6		28 (1)					
Uninjected	—		23 (4)			0 (3)		
Runted	+4		23 (3)			94 (1)		
Uninjected	—		17 (4)			0 (3)		
Runted	+2		42 (4)			100 (1)		
Uninjected	—		17 (6)					
Runted	0		88 (4)					
Isologous cell-injected	−3		12 (2)					0 (1)
Runted	−3		108 (3)					110 (1)
Uninjected	—				0 (1)			
Runted	−6				102 (1)			
Uninjected	—		24 (5)	15 (2)	1 (2)		0 (1)	
Runted	−8		97 (9)	96 (4)	99 (4)		102 (1)	
Uninjected	—	40 (1)	2 (1)	0 (2)		0 (1)		
Runted	−16, −17	98 (4)	114 (1)	106 (7)		91 (1)		
Uninjected	—			5 (4)		0 (6)		
Runted	−34, −38			93 (2)		103 (3)		

* Time of antigenic challenge in relation to cell injection: + (plus) indicates administration of cells after antigen; − (minus) administration of cells before antigen.

‡ Column heads refer to day of bleeding, and entries give mean per cent of surviving phage followed in parenthesis by the number of animals in the group.

§ Values given for 8 hour incubation.

TABLE III

Antibody Production of Normal Adult (A × $C_{57}Bl/1$)F_1 Hybrid and A Strain Mice

Strain	No. of animals	Time following antigen injection	Surviving phage plaques after 2 hrs. of incubation
		days	*per cent*
A	10	10	16.5 (3 to 40)*
(A × $C_{57}Bl/1$)F_1	9	10	21.2 (1 to 55)

* Range in parentheses.

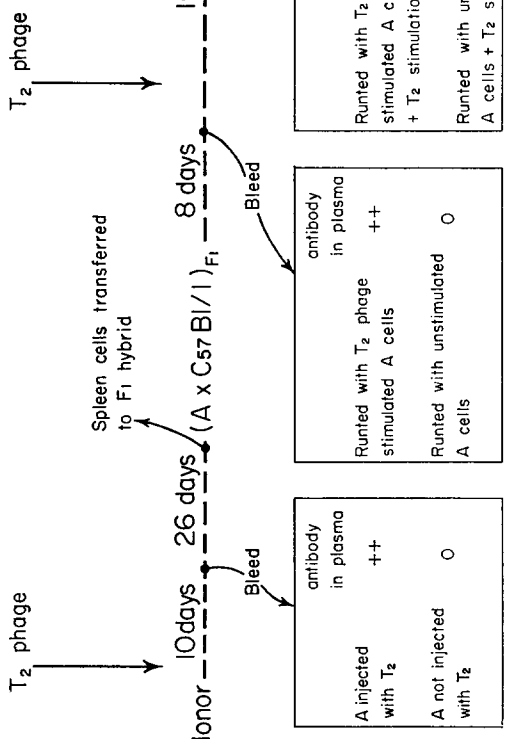

FIG. 5. Effect of prior stimulation of A strain cell donor with bacteriophage T_2 on the response of the runted (A × C_{57}Bl/1)F_1 cell recipients to the same antigen. As shown in the figure, 8 days after cell administration the hybrid recipients had significant levels of phage-neutralizing antibody. When the runted hybrids were themselves stimulated with the same antigen, high levels of phage-neutralizing activity were recorded.

termine whether the immunologic depression of runted hybrid mice would be significantly affected if the parent strain spleen cells were taken from donors previously immunized with the bacteriophage. The plan of the experiment and the results are summarized in Fig. 5.

The prospective A strain donors were injected with T_2 phage, 10^{10} particles, and their antibody response assessed in the usual way on blood samples taken 10 days following injection of antigen. All formed significant amounts of antibody as shown in the figure. Twenty-six days later, these animals were sacrificed and their spleens removed. Each F_1 recipient was given cells from one spleen (130 to 170 million cells), bled 8 days later for antibody assay, and then stimulated with 10^{10} particles of bacteriophage. Appropriate control animals receiving unstimulated parent strain cells were included.

All the animals in this group developed classical runt disease within the month after cell administration. The phage neutralization procedures disclosed significant amounts of antibody in all the recipients of cells from previously immunized donors, whereas controls runted with cells not previously stimulated showed no antibody at this time. When the recipient animals were themselves stimulated with phage, 10-day specimens showed large amounts of antibody as indicated by complete neutralization of the bacteriophage in the test assay within 30 minutes. Control hybrid recipients of spleen cells from parent strain donors not previously stimulated with the antigen did not develop significant antibody.

These observations indicate that, in animals undergoing a graft *versus* host reaction, donor cells previously committed to antigen can survive, continue to function, and even be further stimulated to immunologic response by the same antigen, in contrast to the immunologic unresponsiveness of both donor and host cells in runted hybrids receiving cells from unstimulated donors.

DISCUSSION

These observations are of interest from several points of view. First of all, they provide confirmation in a different immunologic system of the observations of Howard and Woodruff (8) which indicate that animals undergoing runt disease are immunologically defective. When the bacteriophage T_2 system of immunologic stimulation and assay was used, this defect appeared to be severe. The immunologic deficiency was produced in both immunologically tolerant hosts and in genetically tolerant hosts following injection of cells capable of eliciting graft *versus* host reactions. The damage to the antibody-producing system could be most readily demonstrated when the antigenic challenge was provided following injection of the spleen cells designed to produce homologous disease. However, even in animals injected with the runt-producing spleen cells several days subsequent to antigenic challenge, a suppressive effect on

immunologic capacity was demonstrated in the assays of serum antibody on blood samples taken 31 to 40 days following antigenic challenge.

Billingham (13) postulated that the gross deficit of lymphoid tissue in the host produced by graft *versus* host reaction may be responsible for fatalities in mice undergoing runting. One possible basis is that the animals, being immunologically defective, are more susceptible to infections which are ultimately lethal. Surprisingly, however, most studies have failed to implicate infection by demonstrable agents (3, 14, 15). In this regard, the mice undergoing homologous disease resemble mice thymectomized in the neonatal period (16). In both groups of animals the lymphoid tissues show gross morphologic deficiencies late in the disease. Growth is defective, and hunched posture is characteristic. Of particular interest is the observation, implicit in this account as well as the prior study of Howard and Woodruff, that early in the course of homologous or runt disease produced in appropriately receptive hosts after injection of immunologically competent foreign cells immunologic inadequacy is evident at the time when the lymphoid tissues of the recipient are not anatomically deficient but rather show evidence of intense proliferative activity (1, 17). It would seem from the observations presented here that the lymphoid tissues of the recipient host are damaged with respect to immunologic responsiveness at a time when they are capable of a high degree of proliferative activity.

Somewhat paradoxical in this regard is the difference observed in this system in which an immunologic analysis was used and in the system studied by Cooper and Howard (18) in which capacity to deal with infection with pneumococci and salmonella was studied. The latter investigators found that animals injected with organisms during the period of maximal lymphoreticular proliferation in the graft *versus* host reaction survived longer and, with the pneumococcal infection, in much higher percentage than did control mice. In our studies, the proliferative period of the graft *versus* host reaction seemed to be associated with almost complete immunologic failure to the antigen used. Perhaps vigorous phagocytosis in the infections studied by Cooper and Howard was sufficient to provide the protection observed in spite of inadequate immune response, or perhaps the immune response is affected to a much lesser degree with other antigens than with the T_2 phage as might be inferred from the earlier studies of Howard and Woodruff (8). Whatever the explanation of this interesting paradox, further studies will be essential to its analysis.

Further, even though the host lymphoid cells become defective as a consequence of the graft *versus* host attack, this does not explain the failure of the runted host to respond by virtue of the immunologic competence of the parent cells injected into the F_1 host and theoretically under no immunologic attack. If clonal selection is operating as it should in theory, some of the donor cells of the chimera should retain the capacity to react to the T_2 antigen. These experiments further demonstrate that the runted animals are capable of responding

after having been injected with preimmunized donor cells, an observation which establishes the adequacy of the general environment of the runted animal to support antibody synthesis. Further, since the runted animals are capable of responding after having been given preimmunized cells, it seems unlikely that the immunologic incompetence of the runts is due to lack of immunologically competent donor cells *per se.*

A possible explanation of the response of these immunologically runted animals is that the relatively limited number of injected parental cells might be totally committed to the tissue antigens of the host and by virtue of this commitment, incapable of responding to third party antigens following establishment of this commitment. Alternatively, it might be postulated that only those donor cells capable of responding to host antigens undergo proliferation which is essential to survival of the cells under these conditions, whereas the unstimulated cells do not proliferate, cannot compete for location or nutriment, and are lost for future antigenic stimulation. It may be that the intense immunologic challenge provided by the host stimulates all the available immunologic cells of the donor. If immunologic commitment exists in reality, it could be considered as substantial evidence against the clonal selection theory of antibody production.

It seems reasonable to postulate that if a phenomenon such as immunologic commitment does indeed exist, tolerance without the risk of runt disease might be produced by inducing proliferation of appropriately stimulated immunologically committed cells. Further, sufficiently intense antigenic stimulation prior to harvest of the cells might interfere with the capacity of spleen cells to induce graft *versus* host reactions. Studies to test these hypotheses are in progress.

Whether immunologic commitment exists or not, it seems to us that these studies establish that the immunologic attack of the immunologically competent donor cells interferes with capacity of host tissue to respond to an unrelated antigen. The concept of immunologic commitment is an intriguing one, and is currently under intensive study in these laboratories.

SUMMARY

1. Adult (A $\times$ C_{57}Bl/1)F_1 hybrids regularly show runt disease when injected with adult spleen cells from A strain donors. This also occurs when A strain spleen cells are administered to adult C_3H mice made tolerant of A strain tissue in the neonatal period.

2. Mice undergoing the graft *versus* host reaction fail to form antibodies to an intraperitoneal challenge of T_2 bacteriophage. This phenomenon was observed well before any of the other overt signs of runting had occurred. Further, inhibition of antibody production to T_2 phage by graft *versus* host reaction initiated at an interval following antigenic stimulation is demonstrated.

3. The basis for the immunologic incompetence of the host with respect to T_2 phage is presumed to be the attack of immunologically competent donor cells on the lymphoid cells of the recipient.

4. The failure of the injected parent strain cells to respond to the antigen used may imply immunologic commitment of these cells.

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