Immune Competence of Germ-Free Rats
I. Increased Responsiveness to Transplantation and Other Antigens[1] (35412)

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(Introduced by M. E. Shaffer)

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How is it that two genetically identical animals usually generate comparable immune responses when one animal has only one-third the amount of lymphoid tissue present in the other? A review of the literature suggests this to be the situation when the immune responses of germ-free (GF) animals are compared to those of similar conventional (CV) animals.

The immune responses of GF animals have been reported to be roughly equivalent to those of similar CV animals (1–3, 14), despite the fact that the lymphatic system of GF animals has been repeatedly observed to represent a smaller porportion of body weight than that of CV animals (4–6). Olson and Wostmann established that GF mice have about one-third the number of lymphoid cells present in comparable isogeneic CV mice. Actually, GF mice have only one-twelfth the number of primitive lymphocytes "of the type usually associated with antibody formation" (6).

These observations appear paradoxical and suggest that the immune system of the GF animal is in fact more competent than that of the CV animal. (Perhaps more efficient or more responsive would be more precise terms.) The present data document the increased immune competence of the GF rat.

Methods. Germ-free rats. Isogeneic, germ-free ACI, Buffalo, and Fisher rats were studied. They were derived by cesarean section from the conventional isogeneic strains by the Department of Vivarial Science and Research of Tulane University. Animals were reared in flexible film, plastic isolators. Their genetic integrity was maintained by continued brother to sister mating. Continued isogenicity was monitored by periodic reciprocal skin isografts. These included GF $\rightleftarrows$ CV as well as GF $\rightleftarrows$ GF. The continued axenic state was proven by weekly bacteriologic cultures of stool, bedding, food, and water. They were fed a special formula autoclaved diet (Purina 5010C).[4]

Skin grafts. Circular, 1.75 cm^2, full-thickness skin grafts were performed as described by Calnan and Fry (7) except that no dressing was applied. A graft was considered dead when over 50% of the surface area had escharified. Punch biopsies were frequently performed and prepared for microscopic study.

Immunization. Some animals were immunized by a single injection of *Escherichia coli* serotype 04 grown in trypticase soy broth (BBL) at 37°, washed, heat-killed, and suspended in saline. The suspension was emulsified in an equal volume of incomplete Freund's adjuvant and injected into the foot

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TABLE I. Skin Allograft Survival in CV[a] and GF[b] Rats.

Donor	Recipient	Condition	N^c	MST[d]	SD	p
Fisher	ACI	CV	6	9.5	0.8	0.001
		GF	13	5.3	0.2	
ACI	Fisher	CV	11	10.6	1.0	0.2
		GF	5	7.8	2.8	
	Buffalo	CV	5	9.8	0.5	0.05
		GF	5	8.0	0.7	
Buffalo	ACI	CV	5	11.0	0.7	0.02
		GF	4	8.0	0.8	

[a] Conventional.
[b] Germ free.
[c] Number of animals.
[d] Mean survival time (days).

pads. The immunizing dose was calculated from the known dry weight of the suspension.

Some animals were immunized with a single intraperitoneal injection of 0.5 ml of 2% suspension of thrice-washed sheep erythrocytes in buffered saline.

Mixed lymphocyte reactions. These were performed as described by Colley and DeWitt (8).

Microhemagglutination. Titrations were performed in microtiter plates as described by Clarke and Casals (9).

Lymphocytotoxicity. A microdroplet assay was adapted from Terasaki and McClelland (10). Rat lymph node lymphocytes and guinea pig complement were used.

Bacterial agglutination. "H" and "O" antigens for agglutination tests were prepared according to standard methods (11). The tests were performed in disposable trays with U-shaped wells (Linbro), using microtiter equipment and standard conditions of incubation. End points were determined by noting the presence or absence of clumps, using a 5× hand lens and oblique light, after resuspension of cells with a glass rod.

Jerne plaque. This technique was performed as originally described (12) except for the following modifications. Plastic 50 × 12-mm petri dishes were used with 3 ml of base agar composed of 1.4% ionagar 2 in 2× Hanks' medium. The dilute agar layer, 0.7 ml of 0.7% ionagar 2, was mixed with 0.1 ml of 20% washed sheep erythrocytes and 0.1 ml of lymphocyte suspension containing 4, 2, or 1 × 10^6 lymphocytes. One ml of 1:5 guinea pig complement in Hanks' medium was added as the final overlay. The lymphocyte suspensions consisted of a pool of all the splenic and lymph node lymphocytes recovered from a single animal. An innoculum of 4 × 10^6 lymphocytes/plate gave best results.

Results. Size of lymphatic system. Preliminary experiments established that our GF rats had less lymphoid tissue than comparable CV rats. Excised spleen and lymph nodes from GF rats provided 30 to 50% of the number of lymphocytes obtained from similar excisions in comparable CV rats.

Skin allograft rejections. First-set skin allografts were performed in 27 GF and 27 CV rats. The following strain combinations were used: ACI ⇄ Buffalo and ACI ⇄ Fisher. Each of these strains is incompatible with the others at the AgB histocompatibility locus. The ACI and Fisher strains are incompatible to each other at the AgC locus in addition. Eighteen skin isografts were also performed. These included the following combinations: GF → GF of each strain, CV → CV of each strain, GF → CV and CV → conventionalized GF stock.

Allografts between strains of GF rats had shorter survival times than allografts between the same strains of conventional rats (Table I). This difference was significant in three of the four strain combinations studied. The greatest difference in mean survival time was 4.2 days, which was observed with allografts from Fisher to ACI rats. There was good

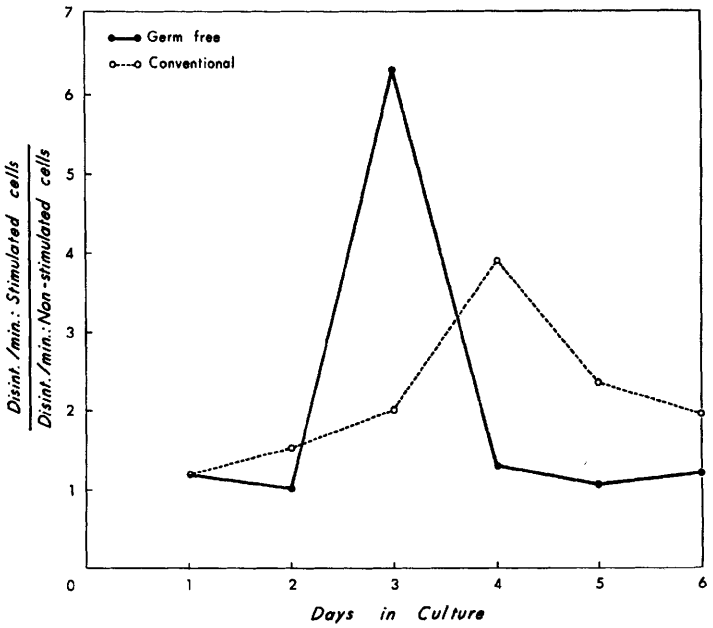

FIG. 1. Mixed lymphocyte reactions of GF-ACI rats stimulated by Fisher cells compared to reactions of CV-ACI rats stimulated similarly.

correlation between gross findings of rejection (stiffness, dark color, and edema) and histologic findings from biopsy material. There was no qualitative histologic difference between rejection in GF animals and in CV animals. The speed of rejection was accelerated in GF animals. Our pathologist was able to distinguish unidentified GF-Fisher to GF-ACI grafts from CV-Fisher to CV-ACI grafts by observing the rapidity of rejection from serial biopsies.

No isografts were rejected prior to sacrifice of the recipients at 3 months.

Mixed lymphocyte reaction (MLR). The ability of GF lymphocytes to respond to allogeneic stimulation was compared to that of CV lymphocytes by the MLR. In these experiments, lymphocytes from GF-ACI rats were stimulated by either GF-Fisher or CV-Fisher nonresponding (irradiated) lymphocytes and the response was compared to the response of lymphocytes from CV-ACI rats stimulated the same way. A triplicate set of all mixtures was harvested daily for 6 days. There was no difference in the stimulation produced by lymphocytes from GF- or CV-Fisher rats. Therefore, data from all mixtures of CV-ACI cells with Fisher cells were pooled and compared to all mixtures of GF-ACI cells with Fisher cells. Consequently in Fig. 1 each point represents the mean of six determininations.

Lymphocytes of GF-ACI rats developed an abrupt and brief burst of mitotic activity 3 days after the exposure to Fisher lymphocytes. Lymphocytes from CV-ACI rats stimulated in the same way developed mitotic activity which later peaked and continued over a longer period of time. The total amount of mitosis was approximately the same (the areas under the two curves are not different) but the GF response was earlier and virtually synchronous.

Serum antiboy response to skin allografts. Serum obtained before grafting and for the first 2 weeks thereafter from each animal was studied for the appearance of hemagglutinins and cytotoxins (Table II).

Neither GF nor CV animals produced detectible hemagglutinins within 2 weeks following skin allografts in two of the strain combinations studied. Recipients in the other two strain combinations did produce hemagglutinins, and GF recipients produced substantially higher titers than did CV recipients. A higher proportion of GF animals de-

TABLE II. Serum Antibody Response to Skin Allografts in CV and GF Rats.

		Mean hemagglutinin titer log-2	No. with cytotoxins
Fisher anti-ACI	CV	4	5/10
	GF	9	5/5
Buffalo anti-ACI	CV	1	0/6
	GF	4	5/5
ACI anti-Fisher	CV	1	1/6
	GF	1	5/12
anti-Buffalo	CV	1	1/5
	GF	1	1/4

veloped cytotoxic sera than did CV animals in all strain combinations studied. This was significant in three of the four.

Response to E. coli *04 immunizations.* Three groups of five GF-Fisher rats were immunized with *E. coli.* One group received 0.05 mg; one group received 0.5 mg; the third group received 5.0 mg. Similar immunizations were performed in 15 CV-Fisher rats. Each animal was bled before immunization and weekly for 3 weeks after immunization. Three animals died during the course of the experiment. Two CV rats were excluded because of significant preimmune anti-*E. coli* titers. Serum from each animal was titered separately. In Fig. 2 are shown the mean titers of the groups, each group consisting of at least four animals. Only anti-O titers are shown, but anti-H titers were similar.

By the end of 1 week the mean titer in each group of GF animals was somewhat higher than mean titers in comparable groups of CV animals. The most impressive difference, however, was between the groups which received 5.0 mg of *E. coli.* CV rats appeared to respond maximally to 0.05 mg of *E. coli* and gave no increase in response to 100 times that amount. In contrast, the response of GF animals continued to rise as the antigen dose was increased.

Jerne plaque experiments. Seven GF-Fisher and seven CV-Fisher rats were immunized with sheep erythrocytes. One GF and one CV animal was sacrificed daily for 7 days and the number of responding lymphocytes determined by the Jerne plaque technique. Figure 3 shows the results. The increase in plaque number above background began a day earlier in GF animals and ultimately rose approximately six times higher than in the CV animals.

Discussion. Few studies of transplantation have been reported in GF animals. GF mice have been observed to reject tumor allografts after neonatal thymectomy and total irradiation although conventional mice die of progressive tumor growth under these circum-

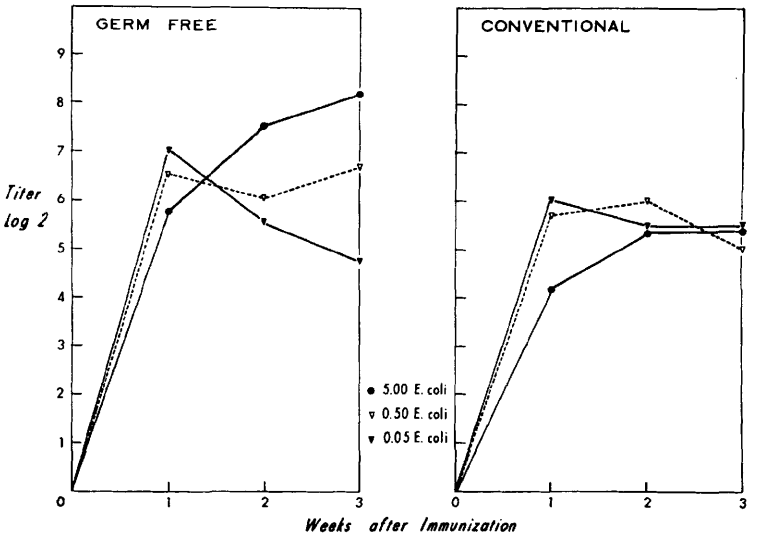

FIG. 2. Serum antibody response of GF-Fisher rats to *E. coli*-04, compared to the response of CV-Fisher rats.

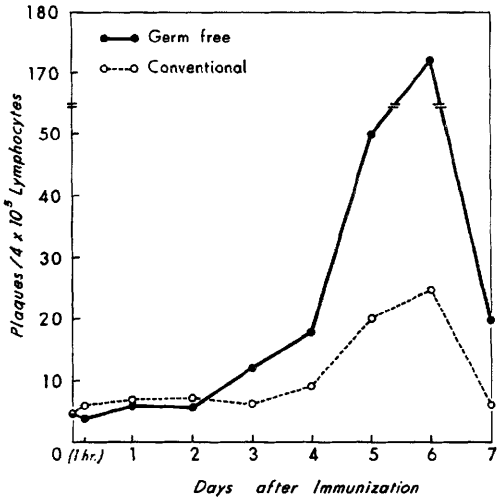

FIG. 3. Cells forming antisheep erythrocyte antibody as determined by the Jerne plaque method. GF-Fisher rats compared to CV-Fisher.

stances (13). GF mice reject skin allografts after neonatal thymectomy but CV mice retain healthy skin allografts until death with wasting disease (14). GF rats in parabioses develop a greater degree of polycythemia or anemia more rapidly than do CV rats (15). We have not found a reference to skin allograft survival between GF rats although no difference in skin allograft survival was noted between GF and CV mice in the study of Miller *et al.* (14).

In our experiments, skin allograft survival was shorter in GF than in CV rats in four different strain combinations, indeed survival time virtually halved in the Fisher to ACI combination.

Comparison of allograft survival times in unmodified recipients would seem a particularly valid way of studying immune competence. An end point of immunopathology produced as a consequence of the total response would seem more reliable than an assay of individual factors which mediate that response. Nevertheless, properly designed experiments should demonstrate *in vitro* correlates which confirm the *in vivo* observations.

Studies with the MLR were performed to reflect delayed-type sensitivity responses. The results demonstrated that lymphocytes from GF animals develop a burst of mitotic activity earlier than do those from CV animals. The fact that the total mitotic activity in both groups was ultimately similar was not surprising. The experimental system provided the necessary environment for a finite amount of metabolic activity beyond which mitosis and cell growth could not be sustained. Presumably these limiting conditions are not present *in vivo*. The experiments therefore offer supporting evidence for increased responsiveness of delayed-type sensitivity in GF rats. The production of hemagglutinins in higher titer and cytotoxins in greater frequency indicates that the increased responsiveness is reflected by immediate-type sensitivity mechanisms also.

Experimental immunizations with *E. coli* were performed to determine if the increased responsiveness of GF animals was unique to transplantation antigens. Studies comparing the humoral response of GF and CV animals to several antigens have been reported with the responsiveness of both types of animals and generally comparable (1–3, 14). However, none of the experiments have analyzed the responses to varying amounts of antigen, nor did they involve use of adjuvant with the immunizations. We elected to take both these steps, the former for completeness and the latter to provide for the slower absorption of antigen. Slower absorption was thought more comparable to the immunization provided by skin allografts. The differences in titers after 1 week were modest but in each group it was higher in the GF animals. The most impressive difference occurred between the two groups which received the largest amount of antigen. The group of GF rats which received 5.0 mg of vaccine produced a clearly higher titer which continued to rise throughout the period of study, compared to its paired CV group.

For the GF animal to generate a greater response than its CV counterpart despite a smaller population of immunologically competent cells, it seemed inevitable that a larger proportion of that smaller population of cells responded to a given stimulus. The experiments using the Jerne plaque technique were designed to test this hypothesis and they confirmed its validity.

The use of Fisher rats may have been fortunate. ACI rats were used in another, less complete, experiment which gave similar results except that the difference between GF and CV plaque formation was threefold rather than sixfold.

Nordin (16) and Bosma *et al.* (17) have compared the number of plaque-forming cells occurring in GF and CV mice following immunization with sheep erythrocytes. In neither study were greater numbers of plaques produced by GF cells. This may be due in part to species differences; however, neither study is strictly comparable to this one. Nordin compared the number of plaque-forming cells obtained from the entire spleen of GF and CV mice without taking into account the difference in cell population. Bosma *et al.* (17) transferred splenic lymphocytes of GF or CV mice into irradiated conventional mice and then determined their response to sheep erythrocytes. The fact that lymphocytes of GF origin were placed in conventional environment may account for their results. The effect of natural immunization from the conventional host upon transferred GF cells may be considerable.

We used mixtures of lymph node and splenic lymphocytes in these experiments; however, this does not account for the results, as other experiments in which only splenic lymphocytes were used gave comparable data.

The mechanism of the increased responsiveness observed is not fully elucidated by these experiments. The fact that it can be demonstrated *in vitro* by the MLR suggests that it is a function of the immune system rather than the general biology of GF life. Some have suggested that the primitive immune system of GF animals is composed of a larger number of uncommitted cells (X-cells or clones) than that of the CV animal. Thus, a larger number of cells are available to respond to any antigen selected at random (1, 14). This suggestion implies that the uncommitted immunocyte is able to respond to a very large number of different antigens, and has implications concerning the selection theories of antibody formation. It would also suggest that when an uncommitted immunocyte becomes committed it is not replaced—a possibility which does not seem likely teleologically. An additional or alternative explanation for the phenomenon may lie in the ability of the GF animal to process antigen. The GF animal is exposed to little natural immunization and might be expected to have more resources available to process antigen more efficiently or more rapidly. This possibility seems plausible and would explain the more rapid as well as stronger responses.

Summary. The ability of GF rats to generate sensitivity responses has been compared to that of isogeneic CV rats. It was demonstrated that GF rats reject skin allografts more rapidly than do similar CV rats. *In vitro* correlates of delayed- and immediate-type sensitivity are produced more rapidly and in greater strength to transplantation antigens. Increased responsiveness was also demonstrated to H and O antigens of *E. coli* and to sheep erythrocytes. This occurs because a larger proportion of the immunologically competent cells of the GF animal's smaller immune system responds to an antigenic challenge.

We suggest, although we have not proven, that this may be due to increased ability to process antigen.

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