THE EFFECTS OF HETEROLOGOUS ANTI-THYMOCYTE SERA IN MICE

III. High Susceptibility of Germfree Mice to the Suppressive Effects of IgG from Rabbit Anti-Mouse Thymocyte Serum

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In the course of studies on the properties of heterologous anti-thymocyte sera (ATS) and its immunoglobulin fractions, a preliminary investigation was made of the effects of ATS on the capacity of spleen cells from germfree mice to mediate graft-vs.-host (GVH) reactions. A quantitative spleen assay (1) had been employed previously to assess the relative potency of different immunoglobulin fractions from ATS (2) and to evaluate the duration of action of ATS in suppressing homograft reactions (3). It was thought that germfree mice might provide better controlled test groups for the investigation of this and other immunologic reactions, since these animals have minimal or no contact with a large variety of antigens present in the "conventional" environment. Germfree mice proved to be surprisingly susceptible to comparatively small amounts of ATS. The present paper describes a detailed investigation of this phenomenon.

Materials and Methods

Animals.—Conventional BALB/cAnN (H-2^d), C57BL/6N (H-2^b) mice and adult New Zealand white rabbits were obtained from the Rodent and Rabbit Production Section, National Institutes of Health. Germfree BALB/cAnN mice, derived from the same genetic nucleus as their conventional counterparts, were obtained from the Germfree Unit, Division of Research Services, National Institutes of Health. The germfree mice were housed in Reyniers' isolators (4, 5) and fed sterilized semi-synthetic diet (No. L-356) (6) and sterile water.

Two groups of conventional animals were placed in isolators at 8 wk of age. One was maintained as were the germfree animals. A second group was fed the same diet, but antibiotics, in the following concentrations, were placed in the sterile drinking water: kanamycin sulfate (as Kantrex, Bristol-Myers, Syracuse, N. Y.) 0.13 mg/ml; neomycin sulfate (as Mycifradin sulfate, Upjohn Co., Kalamazoo, Mich.) 0.3 mg/ml; nystatin (as Mycostatin, Squibb, New York) 50 μ/ml; polymyxin B sulfate (Pfizer, New York) 125 μ/ml.

This effort to sterilize the intestinal tract was only partly successful; *Bacillus proteus* re-

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mained throughout the course of the experiment. Germfree animals were tested for the presence of viable microorganisms by previously described methods (7).

Anti-Thymocyte Sera.—All experiments were performed using antisera described in previous reports. These were prepared in New Zealand white rabbits against Balb/c thymocytes using the method of Levey and Medawar (8). The antisera were absorbed with washed Balb/c erythrocytes (1:20 red cell:ATS ratio) prior to use until hemagglutinins were removed. The IgG fraction of ATS was prepared by means of an ammonium sulfate precipitation of the globulins, followed by separation of the classes of immunoglobulins by G-200 Sephadex chromatography, as previously described (2). This IgG fraction will be referred to as AT-IgG. The purity of the preparation was checked by immunoelectrophoretic analysis with appropriate specific and polyvalent antisera and the concentration determined by spectrophotometry.

The antisera, or AT-IgG, were administered to recipient mice by the subcutaneous route. All injections were made in a volume of 0.5 ml.

Graft-vs.-Host Assay.—A modification (3) of the spleen assay, described by Simonsen (1), was performed. Adult Balb/c mice were injected with either ATS or AT-IgG; at different intervals after treatment, their spleens were removed and a cell suspension prepared. Doses of cells varying from 5×10^5 to 2×10^7 were injected intraperitoneally into newborn C57BL/6N mice. For each cell dose studied, animals from two to four litters were injected; uninjected littermates served as controls. Three cell doses were used in each experimental group. 9 days after grafting of cells, litters were sacrificed and a spleen weight to body weight ratio determined for each group. An index of spleen enlargement was computed by dividing the spleen weight to body weight ratio of the injected animals by that of their untreated littermate controls. As previously noted, there was a linear relationship between the spleen index and the logarithm of the number of grafted cells when spleen cells from normal conventional animals were used (1, 3). Doses higher than 1×10^7 cells caused runting. Therefore, these animals were not included in the normal curve. The observed relationship between cell inoculum and spleen index remained linear when spleen cells from treated animals were tested; the lines in these latter cases were either parallel to or superimposed on that obtained with normal cells. The amount of suppression or hyperreactivity was quantitated by dividing the number of cells from normal mice necessary to produce a given spleen index by the number of cells from treated mice needed to produce the same spleen index. The ratio is constant for all cell doses when the lines are parallel. The "per cent normal reactivity" is this ratio multiplied by 100. A similar formulation has been given by Simonsen (1).

RESULTS

The Effect of ATS or AT-IgG on Graft-vs.-Host Reactions Produced by Cells from Conventional and Germfree Mice.—Indices obtained with live spleen cells from untreated germfree mice fell on a line parallel to, but somewhat lower than that obtained with spleen cells from conventional animals (Fig. 1). The effect of 0.6 mg of AT-IgG on the ability of spleen cells from germfree and conventional mice to evoke a graft-vs.-host reaction is seen in Fig. 1. In conventional mice, a low level of suppression was noted when the assay was performed 2 days after treatment with AT-IgG but not when it was done at later times. In germfree mice a much greater and more prolonged suppression was seen; 65 days after treatment, the reactivity of spleen cells from these mice was only 33% of normal. Spleen cells from germfree mice given 0.6 or 6 mg of normal

rabbit γ-globulin were not suppressed to a significant degree. The maximum amount of suppression produced by 0.6 mg AT-IgG in germfree mice, was

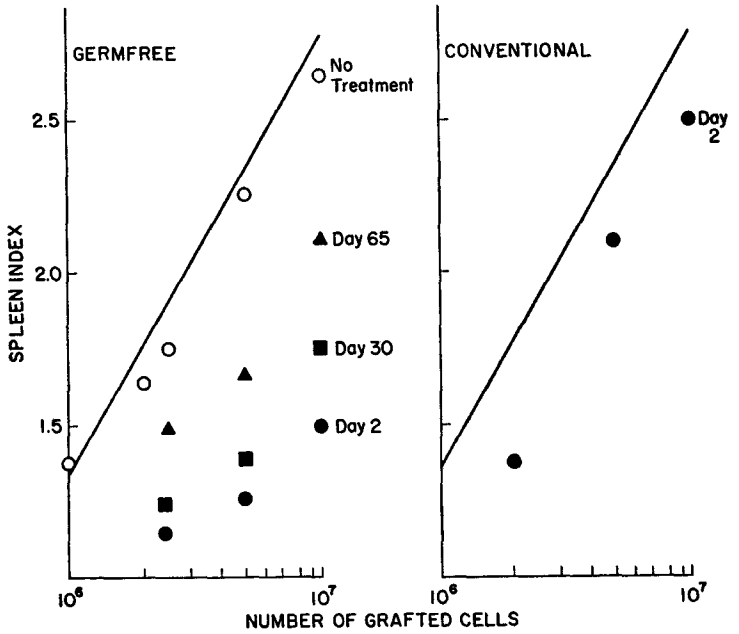

FIG. 1. The effect of a single injection of the IgG fraction of anti-thymocyte serum on graft-vs.-host reactions produced by spleen cells from germfree and conventional mice. Left half: The normal graft-vs.-host reaction that resulted when normal conventional adult BALB/cAnN spleen cells were injected into newborn C57BL/6N mice is shown by the solid black line (3). The reactivity of pools of spleen cells from untreated germfree mice (○) and those treated with a single injection of 0.6 mg of the IgG fraction of ATS at 2 (●), 30 (■), and 65 (▲) days prior to assay are indicated. Each point represents the mean value for 2–4 litters of mice. The spleen index (ordinate) is a measure of the splenomegaly induced by the grafted cells; it was obtained by dividing the spleen weight of grafted cells/100 g body weight by the spleen weight of untreated littermate controls/100 g body weight (3). The abscissa represents the number of grafted cells (logarithmic scale). Spleen indices smaller than 1.3 are not considered to be indicative of significant splenomegaly (1).

Right half: The effect of a single injection of 0.6 mg of the IgG fraction of ATS on the reactivity of spleen cells from conventional mice (●) was assayed 2 days after treatment. The solid line represents the normal graft-vs.-host reaction.

comparable to that produced by 0.5 ml of whole ATS (containing at least 6 mg IgG) in conventional mice.

The rates at which germfree and conventional mice recovered from the effects of ATS or AT-IgG were determined by comparison of the reactivity of their spleen cell populations at various times after injection. The kinetics of recovery

of germfree mice that received 0.6 mg AT-IgG and of conventional mice that received 0.5 ml ATS are depicted in Fig. 2. Germfree mice, as noted above, were seen to be far more susceptible to the action of AT-IgG than their conven-

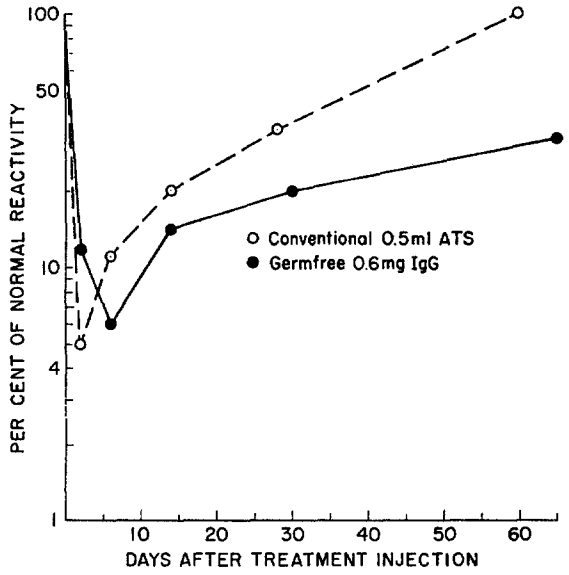

FIG. 2. Comparative effect of anti-thymocyte serum in conventional mice and its IgG fraction in germfree mice. The effect of a single dose of anti-thymocyte serum on the reactivity of spleen cells of conventional mice (O) and of its IgG fraction on the reactivity of spleen cells from germfree (●) mice was assayed by the graft-vs.-host reaction at varying times after treatment. The germfree mice received 0.6 mg of the IgG fraction on day 0 while the conventional animals were given 0.5 ml of ATS. Each point represents the mean value calculated from a graft-vs.-host assay that was done at three different cell transfer doses with 2–4 litters at each dose. The per cent of normal spleen cell reactivity was computed by the formula $\frac{Nu}{Nt} \times 100$ where $\frac{Nu}{Nt}$ is the number of cells from untreated mice divided by the number of cells from treated mice required to result in a given spleen index. The reactivity of normal conventional spleen cells is represented by the 100% value.

tional counterparts. Of note is the roughly straight line relationship between time and the logarithm of the "per cent normal reactivity" when the latter exceeded 15%. This allowed for the calculation of a rate of recovery. An approximately twofold difference was found between the recovery rates of the conventional and germfree mice.

Effect of Diet, Confinement in an Isolator, and Oral Antibiotics on the Susceptibility of Conventional Mice to AT-IgG.—To investigate the possibility that

dietary or other nonspecific environmental factors were responsible for the increased susceptibility of germfree mice to AT-IgG several control experiments were performed. Conventional mice were fed only the germfree diet, sterile water, and were confined in an isolator. After 12 and 54 day periods of isolation,

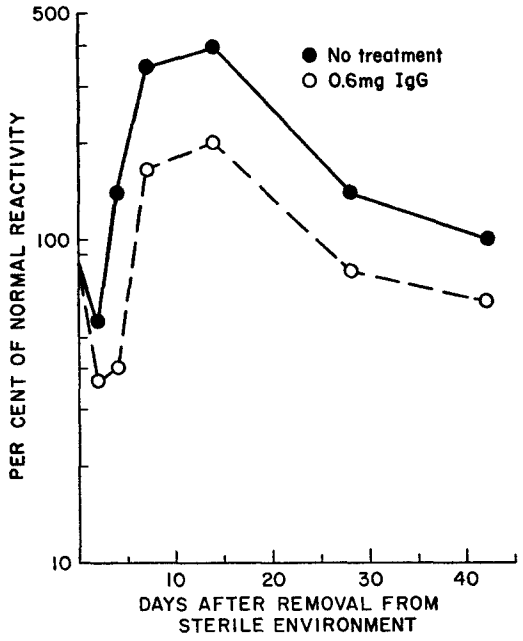

FIG. 3. The effect of the IgG fraction of anti-thymocyte serum on the reactivity of spleen cells of "conventionalized" mice as assayed by the graft-vs.-host reaction. The effect of 0.6 mg of the IgG fraction of ATS on the reactivity of spleen cells of Balb/c mice at 2, 4, 7, 14, 28, and 42 days after their removal from a sterile environment is represented by the lower curve (O- -O). In all cases the treated mice received the IgG fraction 2 days prior to assay (0, 2, 5, 12, 26, and 40 days after conventionalization). The upper curve (●—●) depicts the results obtained when spleen cells from conventionalized mice that did not receive IgG were tested in the graft-vs.-host assay. The per cent of normal reactivity was calculated as described in Fig. 2.

they received 0.6 mg AT-IgG and their spleen cells were assayed 2 days later by the graft-vs.-host method. Spleen cells from untreated, confined mice were also assayed. The reactivity of cells from both groups was identical with that of cells from similarly treated animals raised in the usual animal quarters.

An attempt was made to sterilize the intestinal tract of conventional mice placed in isolators and fed germfree diet. These mice were given an oral antibiotic regimen that included kanamycin, Mycostatin, neomycin, and polymyxin B. Serial stool cultures revealed changes in the quality and density of

the flora; after 1 wk coliform and Gram-positive bacteria were greatly reduced or not detected; after 3 wk *B. proteus* predominated. Quantitative bacterial counts were not done. After treatment periods of 12 and 48 days mice were injected with 0.6 mg AT-IgG and their spleen cells tested 2 days later in the

TABLE I

Litter No.	Inoculum	Spleen indices of individual recipients				Mean spleen index
	× 10^{-6} cells					
1	20	1.10	1.07	1.07	0.92	1.04
2	20	1.22	1.16	1.04		1.14
3	20	1.19	1.07	1.02	1.02	1.08
4	10	1.11	1.04	0.54		0.90
5	10	1.04	0.92	0.80		0.92

Table shows failure of spleen cells from exgermfree Balb/c mice, obtained 14 days after removal of mice from isolators, to produce splenomegaly when transferred to newborn syngeneic recipients. Spleen indices greater than 1.30 represent statistically significant spleen enlargement (1).

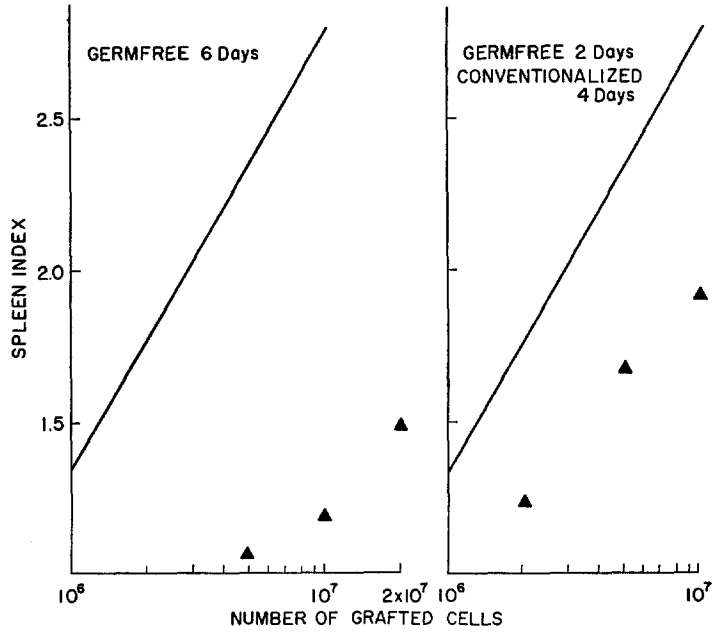

FIG. 4. The effect of conventionalization on suppression by IgG from anti-thymocyte serum in germfree mice. Germfree mice were given a single injection of 0.6 mg of the IgG fraction of ATS and then assayed 6 days after treatment. One group of treated mice was kept germfree for the entire 6 day period (left half). A second group of mice was conventionalized 2 days after treatment and their spleens assayed 4 days later (right half). The solid line represents the normal graft-vs.-host reaction.

graft-vs.-host assay. The combination of this antibiotic regimen, germfree diet, and isolation did not change either the reactivity of the spleen cell population, or their susceptibility to AT-IgG, as determined by this assay.

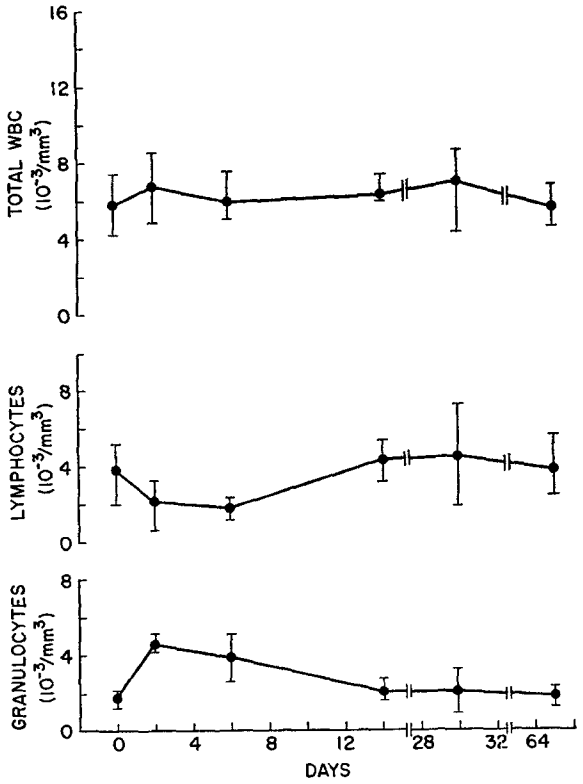

FIG. 5. Effect of a single injection of 0.6 mg of the IgG fraction of anti-thymocyte serum on the peripheral leukocyte counts of germfree mice. Groups of adult germfree BALB/cAnN mice were killed at 2, 6, 14, 30, and 65 days after a single 0.6 mg IgG dose. Total and differential leukocyte counts were performed on individual mice. At each time interval studied the range of counts is indicated by a bracket while the median count is signified by a solid black dot. Leukocyte counts for a group of uninjected germfree control mice are shown as the day 0 values.

Effect of "Conventionalization" of Germfree Mice on the Reactivity of Their Spleen Cells in the Graft-vs.-Host Reaction.—Germfree mice 8–10 wk of age were removed from germfree isolators and placed in a conventional animal room. They were then maintained on diet L-356 and tap water ad lib. At varying times after "conventionalization," groups of these exgermfree mice received 0.6 mg AT-IgG and their spleen cells were tested 2 days later for ability to evoke a graft-vs.-host reaction. Spleen cells from similar groups of untreated conventionalized animals were tested at the same times. The relationship of

time after conventionalization to spleen cell reactivity, for both treated and untreated mice, is given in Fig. 3. After an initial transient hyporeactivity, spleen cells from the untreated mice became hyperreactive. This hyperreactivity

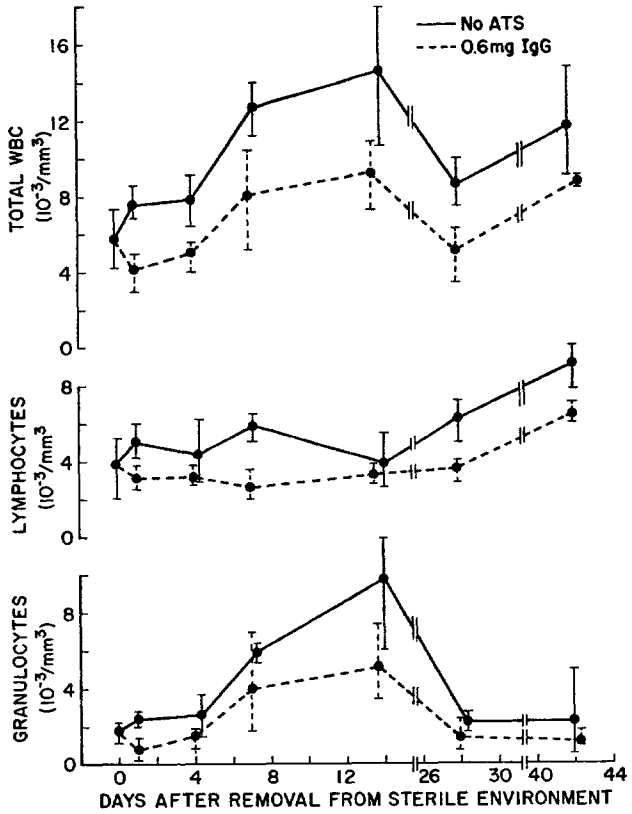

FIG. 6. The effect of a single injection of 0.6 mg of the IgG fraction of anti-thymocyte serum on the peripheral leukocyte counts of conventionalized mice. Groups of germfree BALB/cAnN mice were killed at 1, 4, 7, 14, 20, and 42 days after removal from their sterile isolators. The treated mice (—) were given a single dose of IgG 2 days prior to sacrifice (except the day 1 point when the dose was administered to the mice immediately upon removal from the isolator). The solid line (——) represents conventionalized mice that received no treatment. Total and differential leukocyte counts were performed on individual mice. The range of counts is given within the bracket while the mean count is designated by a solid black dot. Leukocyte counts for a group of uninjected germfree mice are shown as the day 0 values.

was maximum at 14 days, after which time there was a gradual return to normal. The curve for the mice treated with AT-IgG was parallel to that of the untreated group. It is of note that the reactivity of the treated group was approximately 50% of that of the untreated group at each time interval studied.

To eliminate the possibility that the hyperreactivity was entirely nonspecific,

spleen cells obtained 14 days after conventionalization were injected into newborn syngeneic Balb/c mice. No significant splenomegaly was seen when as many as 20×10^6 cells were transferred (Table I).

The Effect of "Conventionalization" on Suppression Initiated in a Germfree Environment.—Germfree mice were given 0.6 mg AT-IgG and then, 2 days later, removed from the isolator. 4 days after "conventionalization," spleens were removed and graft-vs.-host assay was performed. Spleen cells from a group of similarly inoculated germfree mice were also assayed 6 days after treatment. As seen in Fig. 4, the spleen cells from the group maintained germfree showed only 7% normal reactivity, whereas cells from the conventionalized group showed 30% normal reactivity. This indicates that contact with a conventional environment promptly accelerates recovery from the suppressed state.

Leukocyte Counts.—Germfree mice that received a single injection of 0.6 mg AT-IgG had a moderate lymphopenia, which was maximum 6 days after treatment, and a granulocytosis, which was maximum 2 days after treatment. Both total leukocyte and differential counts had returned to normal by day 14 (Fig. 5). The removal of germfree mice from their isolators resulted in marked changes in their peripheral leukocyte counts (Fig. 6). The total leukocyte count rose from the levels of 5800/mm³ seen in a germfree control group to a maximum of 14,400/mm³ on day 14, then fell to 11,600/mm³ on day 42. The lymphocyte count gradually rose from 3800/mm³ on day 0 to 9000/mm³ on day 42 while the granulocyte count increased from 1800/mm³ on day 0 to 9800/mm³ on day 14 and then fell to 2200/mm³ on day 42. The leukocyte counts of conventionalized mice that received 0.6 mg AT-IgG 2 days prior to sacrifice were less than those of their untreated counterparts. In all instances, both the lymphocyte and granulocyte counts were lower in the group of mice treated with AT-IgG. It was noted that the maximum decrease in granulocyte counts resulting from treatment with AT-IgG occurred on day 14, a time at which granulocytosis was at a maximum.

DISCUSSION

These experiments show that conventionally reared and germfree mice differ markedly in their susceptibility to the suppressive effects of AT-IgG. It was previously reported that 0.5 ml of whole ATS was required to reduce the reactivity of spleen cells from conventional mice to 7% of normal (3). These preparations of ATS contained about 12 mg IgG/ml. Furthermore, it was noted that 6 mg of AT-IgG isolated from such antisera did not have a suppressive effect equivalent to 0.5 ml of the unfractionated serum (2). Since 0.6 mg AT-IgG administered to germfree mice was at least as effective as 0.5 ml of whole ATS given to conventional mice, it may be concluded that the AT-IgG was at least 10 times as effective in the germfree as in the conventional animals.

It is unlikely that the susceptibility of germfree mice is the result of some unidentified artifact consequent on confinement in steel isolators or mainte-

nance on a special diet, since similarly maintained conventional animals showed completely normal patterns of reaction to AT-IgG. It was not possible to test conventional animals rendered germfree, since the antibiotic regimen employed did not eliminate *B. proteus*, but it may be concluded that a high degree of modification of the intestinal flora does not affect susceptibility to AT-IgG.

It is known that the size of the total lymphoid mass is smaller in germfree than in conventional animals (9). Although spleen cells from germfree mice were almost as effective in mediating GVH reactions as those from conventional mice, we have no estimate of the size of the total pool of cells capable of mediating these reactions. Thoracic duct (10), bone marrow (11), lymph node (11), and thymus cells (11, 12) participate in GVH reactions. It is also known that there is appreciable traffic of cells among lymphoid organs (13, 14). Only spleens were tested in the present experiments. Furthermore, at least two kinds of cells capable of mediating GVH reactions have been demonstrated in the chicken (15–17), and previously reported data from this laboratory suggested the existence of two such populations in mice differing in susceptibility to AT-IgG (2). It is possible that cells mediating GVH reactions from germfree mice all belong to a population that is particularly susceptible to the action of AT-IgG.

None of the above explains adequately the differences observed in susceptibility to AT-IgG. After only 2 days in a conventional environment, exgermfree mice were no more susceptible than conventional mice. It is most unlikely that the lymphoid mass, or any fraction thereof, increased 10-fold in this short time, especially since the reactivity of spleen cells from untreated animals actually fell during this period. It also should be noted that spleen cells from germfree mice given AT-IgG while in their isolators, then removed to a conventional animal room for 4 days showed an increased reactivity of only fourfold over cells from similarly treated animals kept in the isolators. (See Fig. 4.)

"Pathogen-free" (18) as well as germfree mice lack detectable haptoglobin and probably other constituents of normal plasma. Inoculation of mice (19) or rabbits (20) with bacteria or bacterial endotoxins results in an increase in synthesis of haptoglobin and other plasma proteins within 24–48 hr. α-globulins with ill-defined suppressive effects on immunologic reactions have been described (21, 22). It is conceivable that a plasma component with an opposite activity, i.e. a protective or stabilizing effect on lymphocytes is present in plasma from normal but not germfree mice. This possibility is attractive because the rapidity with which synthesis of many plasma proteins can be induced after exposure to bacteria or their products would explain the prompt loss of susceptibility of germfree mice to AT-IgG after exposure to environmental microorganisms.

The data clearly show a difference in the rate of recovery of germfree mice from the suppressive effects of AT-IgG, and of conventional mice from the suppressive effects of ATS. These two groups were compared since they permitted an estimation of rate of recovery from a similar suppressed level. In

both cases (beginning about 2 wk after treatment) this rate was approximately exponential but was twice as rapid in the conventional group. It has been reported that the rate of turnover of lymphoid cells is slower in germfree than in conventional mice (23), although contrary evidence concerning over-all turnover has been presented by Olson and Wostmann (24). However, the latter investigators have elsewhere presented data which indicates a much more rapid turnover rate for small lymphocytes in conventional mice (25). The evidence is consistent with the hypothesis that lymphoid tissues recover from the suppressive effects of AT-IgG and ATS by regeneration.

The removal of germfree mice from sterile isolators to a conventional animal room transiently but markedly increased the ability of their spleen cells to effect a GVH reaction. The immunologic specificity of the observed state of heightened reactivity is unclear. The production of splenomegaly by these cells was at least directional, in that it occurred in allogeneic but not in syngeneic transfers. It is by no means certain, however, that exposure to environmental antigens sensitized the mice to C57BL/6N tissue. It is known that many antigens are very ubiquitous in nature (26). Kaplan and coworkers (27–29) and others (30-32) have demonstrated a cross-reactivity between bacterial and mammalian antigens. Chase and Rapaport have shown that the inoculation of guinea pigs with certain strains of heat-killed streptococci (33) or staphylococci (34) enabled the animals to reject allografts in an accelerated fashion if grafts were done 11–14 days after injection of the dead bacteria.

It must be kept in mind that the assay employed in the present study is a cell-potency test. Hyperreactivity could be achieved either by (*a*) increasing the number of specifically reactive cells in an inoculum of given size, or (*b*) increasing the capacity to react to allogeneic tissue of a constant number of cells in such an inoculum. The former would represent true immunological sensitization, while the latter should be regarded as an "adjuvant effect." The data presented in this report show that an approximately 50% reduction in reactivity was obtained with 0.6 mg AT-IgG at all times following conventionalization. It would be expected that a fixed dose of AT-IgG would reduce reactivity at proportionately decreasing levels, if the numbers of reactive cells were rising. It appears, therefore, that the numbers of these cells are relatively constant, and that the observed hyperreactivity is the consequence of an adjuvant effect, probably related to the acquisition of a bacterial flora.

Analysis of blood lymphocyte counts after administration of AT-IgG to germfree mice shows return to normal by day 14. The capacity of spleen cells to mediate GVH reactions at this time was about 12% of normal, and indeed did not exceed 33% of normal for the following 51 days. The conclusion drawn previously from a similar analysis in conventional animals (3), that blood lymphocyte counts are a poor index of the state of suppression, is therefore confirmed.

It is also worth noting that AT-IgG lowered blood granulocyte counts during

periods of granulocytosis in exgermfree mice. Such effects on granulocyte levels have not been noted previously when these preparations of AT-IgG (2, 3) or when ATS were administered to animals with a presumably normal rate of granulocyte turnover. The finding indicates that there are probably target cells other than the lymphocyte (such as granulocyte precursors) which will be functionally impaired after treatment with AT-IgG or ATS.

SUMMARY

A quantitative graft-vs.-host (GVH) assay was used to compare the reactivity of spleen cells from germfree (GF) and conventionally reared (CV) mice against allogeneic tissue before and after treatment with rabbit anti-mouse thymocyte serum (ATS) and its IgG fraction (AT-IgG). AT-IgG produced a far greater and longer lasting suppression of this reactivity in GF than in CV mice. Moreover, CV mice recovered from suppression twice as rapidly as did GF mice. In both groups, the rate of recovery was exponential. These results suggest that recovery from the suppressive effects of ATS or AT-IgG was the result of generation of new cells.

Transfer of mice born and initially reared in a conventional animal room to germfree isolators, with subsequent maintenance on the same diet that the germfree mice received, did not change the reactivity of their spleen cells in the assay used nor their susceptibility to AT-IgG. Removal of GF mice to a conventional animal room resulted in a prompt reduction in susceptibility to AT-IgG. The possibility that this might be related to the elaboration of a plasma factor affecting lymphocyte stability was discussed.

Spleen cells taken from GF mice at various times after such "conventionalization" showed a transient but marked hyperreactivity to tissues of the allogeneic recipients. The amount of reduction in reactivity of spleen cells from such mice treated with AT-IgG was always proportional to the activity of spleen cells from comparable untreated mice. It was suggested that the increased reactions evoked should be ascribed to an adjuvant effect rather than to specific immunologic sensitization.

Blood lymphocyte counts correlated very poorly with the state of suppression, confirming previous observations. It was also shown that while AT-IgG had little or no effect on blood granulocyte counts in both GF and CV mice, marked reductions in circulating granulocytes followed administration of AT-IgG during the period of increased granulocytopoiesis that resulted from conventionalization. This demonstrated that AT-IgG can produce functional impairment of target cells other than lymphocytes.

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