REACTIVITY OF LYMPHOCYTES FROM GERMFREE RATS IN MIXED LEUKOCYTE CULTURE AND IN GRAFT-VERSUS-HOST REACTION*

BY H. E. NIELSEN

(From the Institute for Experimental Immunology, University of Copenhagen, DK 2100 Copenhagen Ø, Denmark)

(Received for publication 4 April 1972)

The reason why peripheral lymphoid tissues of nonimmunized animals contain a high proportion of cells responding to strong histocompatibility antigens (H-ag's) of the species (1, 2) is unknown.

One possibility might be that the nonimmunized animals were in fact immunized through cross-reactions between H-ag's and microbial antigens. A few reports in the literature indicate cross-reactivity between staphylococci and H-ag's (3) and streptococci and H-ag's (4, 5).

This hypothesis has been tested previously by comparing the reactivity of germfree (GF) and conventional (CV) animals towards allogeneic H-ag's. The conclusions of the experiments are somewhat conflicting, though the differences found were never very marked in either direction.

| Animal | Authors | GVHR  | Allogeneic MLC |
|--------|---------|-------|----------------|
| Rats   | Wilson and Fox (6) | GF = CV |              |
|        | McDonald et al. (7) | GF > CV |              |
| Mice   | Salomon and Lecourt (8) | GF < CV |              |
|        | Mandel and Asofsky (9) | GF < CV |              |

Wilson and Fox (6) also compared the xenogeneic mixed leukocyte culture (MLC) response of germfree and conventional rats. They found that lymphocytes from conventional rats proliferated when mixed with mitomycin-treated human cells while lymphocytes from germfree rats did not.

In the present report two estimates of the cellular immune reactivity of GF

* This work was supported by The Danish Medical Research Council, and P. Carl Petersen's Foundation.

1 Abbreviations used in this paper: Ag-B, major histocompatibility locus; CDF, cesarean-derived Fischer strain of rats; CV, conventional; F.I., factor of immunisation; GF, germfree; GVH, graft-versus-host; GVHR, graft-versus-host reaction; H-ag, histocompatibility antigen; Hum(m), mitomycin-treated human leukocytes; Ig, immunoglobulin; MLC, mixed leukocyte culture; PHA, phytohemagglutinin.
and CV rats were compared: namely, the reactivity against allogeneic and xenogeneic H-ag's in MLC and graft-versus-host reaction (GVHR).

There was no difference between GF and CV rats in any of these systems. Furthermore, there was the same small increase in GVHR after immunization against a strong H-ag.

**Materials and Methods**

*Rats.*—Inbred male CDF (cesarean-derived Fischer; this strain originated from Fischer 344 11 yr ago. F and CDF mutually accept skin grafts indefinitely) were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. They were allowed to aclimatize for at least 6 wk and were used for the experiments when between 10 and 28 wk of age.

The GF CDF rats were reared in flexible film, plastic isolators. The diet was a radiation-sterilized (5 MR) Rostock mixture enriched before radiation to fulfill the National Academy of Sciences-National Research Council (NAS-NRC) requirements for rats (kindly obtained from veterinarian W. H. Eriksen, The Atomic Energy Commission Research Establishment, Risø, Denmark). The sterility of the isolators was tested two to four times per month by incubation in thioglycollate and Sabouraud's medium (kindly performed by The University Institute for Medical Microbiology, Copenhagen).

The CV CDF rats matched for age were obtained as cesarean-originated, barrier-sustained animals. They were housed in a separate room in an ordinary animal house and were fed Rostock mixture.

The F1 hybrids were all CV. F × BN were used as stimulating cells in MLC. F × BN and F × AS were used as recipients in GVHR. AS × BN were used for immunizations. F and BN differ from each other at the major histocompatibility locus (Ag-B), while F and AS are Ag-B identical.

**Leukocyte Cultures.**—Leukocyte cultures were performed according to Sørensen et al. (10) with the modification that the serum was heat inactivated. Briefly, blood was obtained by heart puncture from rats and by venipuncture from humans. It was defibrinated and sedimented with dextran. The rat leukocytes, which were to be cultured mixed with human mitomycin-treated cells, were washed twice in medium, while those which were mixed with rat cells were not washed. Human and rat leukocytes were incubated with mitomycin C, 25 µg/ml cell suspension, for 20 min and washed three times in medium. The concentration of mononuclear leukocytes was adjusted to 3.0 × 10⁶/ml.

The cells were cultured in 500-µl vol, unmixed or mixed with a one-to-one ratio between responding and stimulating cells, for 88 hr. 300 µl of medium was changed on day 3, the cultures were incubated with thymidine-14C for the last 16 hr, and the activity was determined by liquid scintillation counting.

**Graft-Versus-Host Assay.**—The lymph node weight assay described by Ford et al. (11) was used. Spleen cells and in two instances also lymph node cells were teased apart with forceps, washed twice, and the number of viable nucleated cells counted with the aid of trypan blue. The graft-versus-host (GVH) activity of GF and CV leukocytes was compared both in Ag-B identical recipients (F × AS) and in Ag-B different recipients (F × BN). For the F × BN recipients a parallel line assay was used. Three or in one case two logarithmically spaced cell doses were used. For the F × AS recipients a single cell dose was used. The cells were injected into the hind footpads of 1-3 month old F₁ rats in a volume of 0.10 ml. Two to six recipients were used for each cell dose. The GVH assay was performed 1-5 wk after the last immunization.

**Immunizations.**—(AS × BN)F₁ fetuses and spleens were removed aseptically from conventional rats. They were taken through the lock of the isolator in tightly stoppered bottles and homogenized in the isolator. The germfree animals were injected, and half of the cell
It was taken out of the isolator and injected into CV rats. The injections were given intraperitoneally and subcutaneously.

The immunization scheme was 10–20 × 10⁶ fetal cells with a 2 wk interval followed by two to three injections of spleen cells with a 1–5 wk interval.

**Alloantibody Titration.**—Serum was obtained from GF and CV rats at the time of the GVH assay. Antibodies against the BN antigen were titrated by the dextran hemagglutination method as described in reference 12.

**Blood Leukocyte Counts.**—Performed by the methyl violet acetic acid method.

**Serum Gamma Globulin.**—Measured according to Everhart and Shefner (13) (kindly performed by Robin J. Hill, B.Sc.).

RESULTS

**Sterility Testing.**—The cultures were consistently negative.

**Size of Lymphatic System.**—Table I shows that the number of leukocytes in

| TABLE I | Size of Lymphatic System in Conventional and Germfree CDF Rats |
|---------|---------------------------------------------------------------|
|         | No. of leukocytes per ml blood (× 10⁶)* | No. of nucleated cells per spleen (× 10⁶)† | mg Ig per ml serum‡ |
|         | CV   | GF   | CV   | GF   | CV   | GF   |
| Median  | 7.2  | 2.7  | 139  | 77   | 10.8 | 1.0  |
| Range   | 3.0–11.1 | 2.3–5.6 | 99–205 | 70–81 | 5.4–21.4 | 0.67–2.7 |
| No. of animals | 10  | 8    | 5    | 4    | 4    | 4    |
| Significance of difference between medians§ | P < 0.001 | P < 0.02 | P < 0.05 |

* Both immunized and nonimmunized rats.
† Only nonimmunized rats.
§ Wilcoxon's rank sum-test.

blood and spleen was significantly lower in germfree than in conventional animals. Also the serum immunoglobulin (Ig) concentration was lower in germfree rats.

**Leukocyte Cultures.**—The results are presented in Table II. 12 different germfree rats were used. There was no significant difference between germfree and conventional rats in any of the types of cultures tested.

In allogeneic mixtures the ratio between counts per minute in conventional and germfree mixtures varied between 0.87 and 2.3 in 10 experiments.

In xenogeneic mixtures there are only five comparisons between germfree and conventional rats. In three experiments the rat lymphocytes were clearly stimulated by human mitomycin-treated cells. Again, there was no difference between germfree and conventional rats, so it can be concluded that lymphocytes from germfree rats can be stimulated to blast transformation by mitomycin-treated human leukocytes.
It is not clear why there was no stimulation in experiments 5 and 7. Later experiments with conventional rats suggest that this may be due to the presence of natural antibodies against human cells in some batches of rat serum. It was found that in the same experiment human mitomycin-treated leukocytes stimulated neither human nor rat lymphocytes when suspended in medium with inactivated rat serum, while the same human leukocytes when suspended in medium with fetal calf serum stimulated both human and rat lymphocytes.

The phytohemagglutinin (PHA) response may be higher in germfree than

| Cultures   | Exp. No. | Median | Range | Difference between medians* |
|------------|----------|--------|-------|-----------------------------|
|            | 1 2 3 4 5 |        |       |                             |
| Unstimulated |          |        |       |                             |
| CV CDF     | 0.45 0.45| 0.35 0.43| 0.31 0.23| 0.28 0.28| 0.37 0.37| 0.36 0.25-0.45| n.s. |
| GF CDF     | 0.22 0.31| 0.29 0.44| 0.30 0.66| 0.36 ---| 0.43 0.33| 0.39 0.22-0.65| n.s. |
| Allogeneic MLC |          |        |       |                             |
| CV CDF + F × BN | 4.1 6.0 | 5.9 3.9| 2.6 2.2| 9.3 7.4| 11.9 1.4| 10.4 0.4-11.9| n.s. |
| GF CDF + F × BN | 5.4 5.8 | 2.6 4.3| 2.8 2.6| 7.3 7.5| 10.5 1.4| 10.4 0.4-10.5| n.s. |
| PHA stimulated |          |        |       |                             |
| CV         | 19.3     | 29.2   | 24.5 24.2| 26.1 | 24.5 | 19.3-29.5 | n.s. |
| GF         | 41.1     | 49.3   | 33.3 25.4| 31.2 | 33.3 | 25.4-49.5 | n.s. |
| Xenogeneic MLC |          |        |       |                             |
| CV CDF + Hum(m) | 3.8     | 0.26   | 0.76 1.9| 2.1 5.1| 1.9 | 0.8-2.6 | n.s. |
| GF CDF + Hum(m) | 3.8     | 0.36   | 0.62 1.1| 1.7 4.5| 4.74 | 0.36-3.8 | n.s. |

Controls (F × BN unmixed, CV CDF + CDF(m), GF CDF + CDF(m), CDF(m) + Hum(m)) < 0.55 × 10^-3 cpm.

* Wilcoxon's signed-rank test.
† PHA-3 (Difco Laboratories, Detroit, Mich.) 1 µl/culture.
‡ The same human donor for Exp. No. 3 and 9, different human donors for the other experiments.

Figs. 1 and 2 record the time-course of thymidine uptake in allogeneic and xenogeneic MLC in a single experiment (No. 9, Table II). The kinetics of the response seem to be identical for germfree and conventional rats.

GVHR: F × BN Recipients.—The comparison of leukocytes from unimmunized germfree and conventional rats is shown in Table III. The relative strength is expressed as the potency ratio, which is the ratio between the number of conventional cells to the number of germfree cells required to give the same lymph node enlargement. This potency ratio was not different from 1. The comparison of immune and nonimmune cells is shown in Table IV. The potency ratio between nonimmune and immune cells is called the factor
FIG. 1. Allogeneic MLC (part of Exp. No. 9, Table II). O, conventional CDF + F × BN. X, germfree CDF + F × BN. SEM of triplicate cultures: 10% of mean. Unmixed controls harvested on day 4: <430 cpm.

FIG. 2. Xenogeneic MLC (part of Exp. No. 9, Table II). O, conventional CDF + Hum(m). X, germfree CDF + Hum(m). SEM of triplicate cultures: 10% of mean. Unstimulated controls harvested on day 4: <358 cpm.

of immunization (F.I.). There was no difference between germfree and conventional rats.

F × AS Recipients.—Table V compares the lymph node enlargement caused by immune cells. There was no difference between germfree and conventional donors.
### TABLE III

**GVH Assay Comparing CV and GF CDF Nonimmune Spleen Cells Injected into (F X BN)F₁ Recipients**

| Exp. No. | Potency ratio* | 3 | 1.31 | 1.81 | 0.88 | 0.65 | 1.82 | 0.84 | Geometric mean |
|----------|----------------|----|------|------|------|------|------|------|---------------|
| 1        | 0.76           |    |      |      |      |      |      |      |               |
| 2        | 0.83           |    |      |      |      |      |      |      |               |
| 3        | 0.88           | 1.31| 1.81 | 0.88 | 0.65 | 1.82 | 0.84 | 1.07 |               |

* Number of CV cells per number of GF cells required to give the same lymph node enlargement.

### TABLE IV

**Immune and Nonimmune CDF Spleen Cells Injected into (F X BN)F₁ Recipients**

| Exp. No. | CV Factor of immunization* | GF |
|----------|----------------------------|----|
| 3        | 1.1                        | 1.2|
| 4        | 1.2                        | 0.8|
| 6        | 1.4                        | 1.7|
| 7        | 1.6                        | 1.3|

Geometric mean 1.3 1.2

* Number of nonimmune cells per number of immune cells required to give the same lymph node enlargement.

### TABLE V

**Lymph Node Weight in (F X AS)F₁ Recipients Injected with Immune CDF Spleen Cells**

| Exp. No. | Cell dose | Log mean lymph node weight ± SEM |
|----------|-----------|---------------------------------|
|          | CV        | GF                              |
| 7        | 5.0 × 10⁶ | 0.99 ± 0.10 (3)*                 | 1.15 ± 0.27 (3) |
| 8        | 24 × 10⁶  | 1.17 ± 0.12 (4)                 | 1.13 ± 0.06 (4) |
| 9        | 15 × 10⁶  | 1.30 ± 0.05 (6)                 | 1.35 ± 0.05 (6) |

* The numbers in parentheses indicate the numbers of lymph nodes.

### TABLE VI

**Antibody Titers Against the BN Antigen in Sera from CV and GF Immunized CDF Rats (Dextran Hemagglutination)**

| Exp. No. | CV   | GF   |
|----------|------|------|
| 3        | 1/27 | 1/27 |
| 4        | 1/27 | 1/81 |
| 6        | 1/729| 1/729|
| 7        | <1/729|<1/729|
| 8        | 1/243| 1/81 |
| 9        | 1/81 | 1/243|
Antibody Titers.—Table VI compares the serum antibody titer of the immunized germfree and conventional rats against the BN antigen. There was no difference in the titers.

DISCUSSION

The idea of cross-reactivity between microorganisms and H-ag’s is only supported by a few experimental data. In spite of an extensive search, cross-reactions have only been postulated between certain strains of staphylococci and streptococci and the H-ag’s of a few species (3-5).

On the other hand, there are a large number of observations which seem incompatible with this hypothesis.

Antibodies against the H-ag’s of the species are never detected in unimmunized animals or humans, but they are very readily detected after immunization with allogeneic cells. Also, there is a clear difference between a first-set and a second-set skin graft rejection.

Fetuses of several mammalian species are able to reject allografts from midgestation (14), and human fetal leukocytes from midgestation can react against H-ag’s in MLC (15).

Finally, there seem to be fewer antigen-sensitive cells against xenoantigens than against alloantigens, probably fewer the more distantly related the two species are (16).

The results of the present experiments seem clear cut: there is no difference between the MLC and GVH activity of germfree and conventional rats. Both of these methods estimate predominantly, in the case of MLC maybe exclusively, the proportion of cells sensitive to a strong H-ag. For the comparison to be valid one must assume that for MLC the lag period before proliferation begins is identical, and that the generation time of the dividing cells is identical. Figs. 1 and 2 suggest that both of these assumptions have been met.

For the GVHR one must assume that the time-course of the lymph node enlargement is identical. This has not been tested, but the tempo of the enlargement is the same whether immune or nonimmune cells are used (12). Furthermore, one must assume that the percentage of lymphocytes in the spleen cell suspensions are identical. Differential counts in the counting chamber showed no difference between germfree and conventional rats.

The allogeneic MLC response was identical for germfree and conventional rats. This is in accordance with the findings of Wilson and Fox (6) using the same rat strain as responder, but in disagreement with the findings of McDonald et al. (7), who used the AC strain as responders in a single experiment, and found that lymphocytes from germfree animals gave the highest MLC response.

Also the xenogeneic MLC response failed to show any difference between germfree and conventional rats, but the number of experiments is too small to allow any firm conclusion on quantitative differences. The finding is in contrast
to that of Wilson and Fox (6), who found that only lymphocytes from conventional rats were reactive to xenogeneic cells.

The effect on cell-mediated immunity of immunization of germfree animals against allogeneic cells has never previously been tested. The small increase in GVH activity after immunization against BN supports the conclusion of the MLC experiments, and of the experiments comparing GVH activity of non-immune germfree and conventional cells: that the proportion of antigen-sensitive cells in unimmunized rats is as high in germfree as in conventional animals. The humoral antibody response towards the BN antigen was identical in germfree and conventional rats. This is in accordance with the finding that the humoral antibody response of germfree animals to several other antigens shows only minor deviations from that of conventional animals (17).

The F.I. after immunization against the weak H-ag AS cannot be estimated precisely, because only immune cells have been injected in single doses. About $25 \times 10^6$ nonimmune spleen cells are needed to give a lymph node weight of 10 mg (12). There is no difference between the GVH activity of conventional and germfree rats after immunization against the weak AS antigen.

The possibility has not been ruled out that antigenic stimulation of germfree rats through dead microorganisms in the food and bedding material, dietary protein, and eventually undetected virus infections could suffice for cross-immunization against H-ag's, but this seems improbable. A reduction in the exposure to environmental antigens, sufficient to cause a large reduction in the size of the lymphoid system and in the Ig concentration in serum, would also be expected to reduce the level of immunization through cross-reactions.

It seems, therefore, reasonable to conclude that the cellular response against allogeneic and xenogeneic H-ag's represents a true primary response.

SUMMARY

The reactivity of lymphoid cells from germfree and conventional CDF rats were compared in mixed leukocyte culture (MLC) with both allogeneic rat leukocytes and with human leukocytes as stimulating cells, and in a graft-versus-host (GVH) assay. There was no difference between germfree and conventional rats in any of these systems. Furthermore, the increase in GVH activity after specific immunization against a strong histocompatibility antigen was small in both cases. The findings are incompatible with the hypothesis that the high proportion of antigen-sensitive cells against strong histocompatibility antigens is caused by cross-reactions between microbial antigens and histocompatibility antigens.

Bodil Nielsen is thanked for excellent technical assistance.

REFERENCES

1. Nisbet, N. W., M. Simonsen, and M. Zaleski. 1969. The frequency of antigen-sensitive cells in tissue transplantation. J. Exp. Med. 129:459.
2. Wilson, D. B., J. L. Blyth, and P. C. Nowell. 1968. Quantitative studies on the mixed lymphocyte interaction in the rat. III. Kinetics of the response. J. Exp. Med. 128:1157.

3. Rapaport, F. T., and R. M. Chase, Jr. 1965. The bacterial induction of homograft sensitivity. II. Effects of sensitization with staphylococci and other microorganisms. J. Exp. Med. 122:733.

4. Chase, R. M., Jr., and F. T. Rapaport. 1965. The bacterial induction of homograft sensitivity. I. Effects of sensitization with group A streptococci. J. Exp. Med. 122:721.

5. Hirata, A. A., and P. J. Terasaki. 1970. Cross-reactions between streptococcal M proteins and human transplantation antigens. Science (Wash. D.C.). 168:1095.

6. Wilson, D. B., and D. H. Fox. 1971. Quantitative studies on the mixed lymphocyte interaction in rats. VI. Reactivity of lymphocytes from conventional and germfree rats to allogeneic and xenogeneic cell surface antigens. J. Exp. Med. 134:857.

7. McDonald, J. C., G. Zimmerman, R. B. Bollinger, and W. A. Pierce. 1971. Immune competence of germfree rats. I. Increased responsiveness to transplantation and other antigens. Proc. Soc. Exp. Biol. Med. 136:987.

8. Salomon, J. C., and J. C. Lecourt. 1966. Studies on homologous disease using germfree mice. Proc. Soc. Exp. Biol. Med. 122:540.

9. Mandel, M. A., and R. Asofsky. 1969. 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. J. Exp. Med. 129:1203.

10. Sørensen, S. F., P. Bildsøe, and M. Simonsen. 1971. Effect of strong and weak histocompatibility antigens on the mixed lymphocyte culture reaction in rats. Acta Pathol. Microbiol. Scand. Sect. B Microbiol. Immunol. 70:475.

11. Ford, W. L., W. Burr, and M. Simonsen. 1970. A lymph node weight assay for the graft-versus-host activity of rat lymphoid cells. Transplantation. 10:258.

12. Ford, W. L., and M. Simonsen. 1971. The factor of immunization in the rat. The effect of allogeneic immunization on graft-versus-host activity. J. Exp. Med. 133:938.

13. Everhart, D. L., and A. M. Sheiner. 1966. Specificity of fish antibody. J. Immunol. 97:231.

14. Silverstein, A. M. 1967. Ontogenesis of the immune response. In Comparative Aspects of Reproductive Failure. K. Benirschke, editor. Springer Publishing Co. Inc., New York. 392.

15. Ceppellini, R., G. D. Bonnard, F. Coppo, V. C. Miggiano, M. Pospisil, E. S. Curti, and M. Pellegrino. 1971. Mixed leucocyte cultures and HL-A antigens. I. Reactivity of young fetuses, newborns and mothers at delivery. Transplant. Proc. 3:58.

16. Lafferty, K. J., and M. A. S. Jones. 1969. Reactions of the graft-versus-host (GVH) type. Aust. J. Exp. Biol. Med. Sci. 47:17.

17. Wostman, B. S. 1968. Defence mechanisms in germ-free animals. I. Humoral defence mechanisms. In The Germfree Animal in Research. M. E. Coates, editor. Academic Press, Inc., New York. 197.