INDUCTION OF TOLERANCE TO SHEEP RED BLOOD CELLS*

BY TRAUTE-HEIDI ANDERSON,† JOAN ROETHLE, AND ROBERT AUERBACH
(From the Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706)
(Received for publication 28 September 1972)

Of all the antigenic material used in immunological studies perhaps none has been
as widely used in recent years as has the sheep erythrocyte. It has been the sheep red
blood cell (SRBC) which has served so frequently as an indicator of serum agglutinins
and hemolysins, as a means of quantitation of antibody-forming cells by rosette forma-
tion or by plaque assay, for delineation of both 19S and 7S antibody-secreting cells,
for induction of immune responses in vitro in organ culture and in cell suspension
systems, for studies of maturation of immune competence, and for the basic work in-
volving cell collaboration in immunity.

Unresponsiveness to SRBC can be induced both in neonatal (1) and adult (2)
animals by a prolonged injection schedule with massive doses of SRBC. More gen-
erally, unresponsiveness has been induced by treatment with antigen in combination
with cytotoxic agents such as cyclophosphamide (3–8). Neither method of induction of
tolerance seems entirely desirable, however. Multiple injections of SRBC activate
antibody synthesis and set into motion complex antibody-mediated feedback systems,
while drug-induced tolerance involves both specific killing of antigen-sensitive cells
as well as a general depressive effect on the general lymphocyte population.

It was our hope to obtain tolerance to SRBC in a manner which did not
knowingly involve cell destruction, and which did not knowingly involve activa-
tion of typical antibody formation against SRBC. We felt that if we could
obtain tolerance in such a manner we might more closely approximate the type
of tolerance significant in the normal development of immunity versus unres-
pponsiveness. In analogy to studies with protein antigens (e.g. aggregated vs.
disaggregated forms of human gamma globulin; cf. references 9 and 10 for re-
view) a number of investigators have attempted to use solubilized SRBC com-
ponents for induction of tolerance (11–13). However, in these studies the tolero-
genic material was initially antigenic and could therefore be assumed to induce
tolerance in the same manner as massive doses of intact SRBC. The experiments
which we present involve simple saline extraction of hemolyzed SRBC followed
by centrifugation to obtain a membrane-free preparation. Our material appears
to be nonantigenic, yet significantly reduces the subsequent specific response to
SRBC.

* Supported in part by grant GB 6637X from the National Science Foundation.
† The initial phases of this study were presented as a Master's Degree dissertation of
T.H.A. at the University of Wisconsin.
Materials and Methods

Animals.—Inbred BALB/CAn mice from our laboratory and additional BALB/CJax animals purchased from Jackson Laboratory, Bar Harbor, Maine, were used in our initial studies. Subsequently, BDFI mice (C57/BL6 × DBA/2) obtained from ARS-Sprague Dawley were used with equivalent results. Experiments were carried out on 2-3-months old male mice.

Erythrocytes.—SRBC and horse red blood cells (HRBC) were obtained from specific donor animals as a 50% suspension in Alsever’s solution. Erythrocytes were washed twice in physiological saline and were resuspended in fresh saline at appropriate concentrations.

Preparation of Hemolysate Supernatant.—2 ml of packed erythrocytes were subjected to hypotonic lysis with 6 ml of glass-distilled, demineralized H2O, the suspension was centrifuged at 1000 g for 10 min to remove leukocytes, and the supernatant centrifuged at 34,000 g for a minimum of 200 min at 4°C. The supernatant obtained from this centrifugation was readjusted to 0.9% salinity with sodium chloride and filter sterilized through 0.2 or 0.45 μ Millipore filters under pressure. Material was normally used within 1 wk of preparation. In later experiments the filtrate was further passed through 50 μm pore filters without apparent loss of activity. In addition, prolonged storage at −20°C did not adversely affect the preparation.

Assay for Immune Response.—Plaque assays were performed by use of the liquid monolayer assay of Cunningham and Szenberg (14). Indirect plaque assays were carried out by addition of 0.025 ml of rabbit anti-mouse gamma globulin (Difco Laboratories, Inc., Detroit, Mich.), 1/50 dilution, using duplicate test material. Adjustment for quenching of 19S plaques proved unnecessary because of the low level of 19S plaques. Agglutination tests were carried out on serial twofold dilutions of serum prepared from blood obtained from the retro-orbital sinus.

Cell Counts.—Cell counts were obtained either by use of a hemacytometer or with a Nuclear Chicago Particle Size Analyzer (Nuclear-Chicago, Des Plaines, Ill.) (Coulter principle) appropriately corrected for dead time and coincidence.

RESULTS

Tolerizing Ability of SRBC Hemolysate Supernatant (SHS).—Adult BALB/C mice were given daily intravenous injections of 0.2 ml of SHS for 7 days; they were challenged with an immunizing dose of 2.5 × 10⁶ SRBC on day 8 and assayed 4 days later. The results, shown in Table I (group A) indicate that this treatment reduces the response to 25% of normal. Parallel experiments indicated, moreover, that the reduction was specific, since this treatment did not reduce the ability of animals to respond to horse red blood cells.

An attempt was made to render animals more completely tolerant by increased number of injections (Table I, group B) or by increasing the dose of a single injection (Table I, group C and other experiments) but no further increase in the amount of suppression was obtained. To examine the length of time that SHS treatment could suppress the immune response to SRBC, animals were tested at varying times after completion of treatment. The results (Table I, group D) indicate that tolerance remained stable for at least 1 month.

In order to facilitate experimentation we next examined the effects of intraperitoneal injections of SHS. We found that seven daily injections of 0.4 ml of SHS intraperitoneally were at least as effective at reducing the response to
SRBC as were intravenous injections. Moreover, five daily injections were ade-
quate; with four injections, however, significant but not maximal reduction in
responsiveness was observed.

Because of the more ready availability of BDF1 mice we tested the effect of
SHS on such animals. We found that BDF1 mice responded at least as well to
the tolerizing effect of SHS as did BALB/C mice (Table II). For most subse-
quent experiments, therefore, we adopted the procedure of treating BDF1 ani-
mals with five intraperitoneal injections of 0.4 ml of SHS, followed after a few
days by challenge with $5 \times 10^8$ intact SRBC, and assayed for plaque-forming
cells (PFC) 4 days after challenge.

**Specificity of Induced Unresponsiveness.**—To examine more critically the
question of specificity of tolerization, reciprocal experiments were carried out
using horse red blood cell hemolysate supernatant (HHS) as well as SHS. Ani-
mals were treated either with SHS, with HHS, or with both preparations, and
subsequently challenged with either HRBC or SRBC. The results are shown in
Table III. It can be seen that HSH reduced the response only to HRBC, just
TABLE II
Effects of Graded Doses or Varied Numbers of Intraperitoneal Injections of Sheep Erythrocyte Hemolysate Supernatant (SHS) on Response of BDF1 Mice to SRBC

| Treatment | No. of animals | PFC/spleen × 10^4 ± se (range) | Control | PFC/10^4 |
|-----------|----------------|-------------------------------|---------|-----------|
| - SRBC    | 8              | 0.2 ± 0.5 (0-0.5)              | 0       | 0.1       |
| + SRBC    | 8              | 106.9 ± 11 (72-155)            | 100     | 34.8      |
| 1 × SHS   | 0.4            | 73.8 ± 20 (23-201)             | 69      | 30.1      |
| 2 × SHS   | 0.4            | 44.6 ± 13 (7-123)              | 42      | 18.5      |
| 3 × SHS   | 0.4            | 32.0 ± 7 (12-68)               | 30      | 20.2      |
| 4 × SHS   | 0.4            | 18.8 ± 4 (1-43)                | 18      | 8.3       |
| 5 × SHS   | 0.4            | 8.2 ± 1 (1-18)                 | 8       | 4.0       |
| 5 × SHS   | 0.2            | 27.4 ± 8 (11-80)               | 26      | 12.6      |
| 5 × SHS   | 0.1            | 40.5 ± 6 (6-60)                | 38      | 24.4      |
| 5 × SHS   | 0.05           | 76.0 ± 15 (55-170)             | 71      | 26.3      |

TABLE III
Specificity of Tolerization with Erythrocyte Hemolysate Supernatant

| Treatment | SRBC challenge | HRBC challenge |
|-----------|----------------|----------------|
|           | SRBC assay     | HRBC assay     |
| None      | 47.6           | 0.4            |
| SHS       | 2.5            | 0.2            |
| HHS       | 45.7           | 1.4            |
| SHS + HHS | 1.4            | 0.3            |

* Four animals per group.

as SHS influenced only the reaction against SRBC. Treatment with both SHS and HHS impaired the response to both antigens.

Immunogenicity of SHS.—Preparations of SHS were routinely checked for antigenicity by injecting 0.4 ml of the material into adult mice and examining spleens from these animals for PFC 4 days after injection. On occasion a preparation was found to be mildly antigenic, presumably due to contaminant material. Such a preparation was excluded from further use. Most preparations were not antigenic by this test.

We next examined the possibility that SHS might prove immunogenic at doses other than those used in our tolerance experiments. We therefore injected SHS in amounts ranging from 0.001 to 0.8 ml intraperitoneally into test animals whose spleens were subsequently examined for PFC. In no instance were the number of PFC in excess of background; indeed the data were suggestive of a decrease in PFC, but this point was not pursued to reach a level of significance.

To ascertain that the route of injection was not critical, 0.1–0.4 ml of SHS was injected via the lateral tail vein and spleens were subsequently examined.
for both direct (19S) and indirect (7S) PFC. Again there was no indication of immunogenicity.

To determine if SHS could become immunogenic when administered in adjuvant, two groups of six animals were injected with 0.2 ml of a 1:1 mixture of Freund’s complete adjuvant with either HSH (experimental) or saline (control). The spleens of both groups assayed after 4 days did not differ significantly from each other in the number of PFC, although the number of observed “background” plaques in both groups rose to an average of 1500 PFC/spleen in contrast to uninjected animals which routinely give background levels of 300-500 PFC/spleen. Cells of the mesenteric and inguinal lymph nodes also showed no response to SHS in adjuvant.

**DISCUSSION**

Our experiments show that animals can be made substantially less responsive to heterologous erythrocytes by a nonimmunogenic red blood cell hemolysate supernatant fraction, without the addition of lympholytic or cytotoxic agents. It appears to us that this presents us with a situation analogous to that first described by Dresser (16) for protein antigens, and which has been so valuable in analysis of questions of immunological unresponsiveness (cf. reference 10). Clearly, further characterization of the tolerogenic supernatants is required, and initial attempts at purification of our material are encouraging.

We feel that tolerance produced by fractions obtained from hemolyzed erythrocytes are more likely to approximate naturally occurring tolerance than is tolerance produced with the aid of cytotoxic drugs. For example, immunoincompetent animals such as the neonatal mouse do clear SRBC from the circulation. Nonimmunological clearance could well lead to the production of tolerogens which might effectively diminish the subsequent response to SRBC. Moreover, if one considers the heterologous erythrocyte as a cell rather than as an antigen one can readily envision a chronology of development that, by this mechanism, leads to restriction of humoral responses directed against cellular self-antigens.

**SUMMARY**

Adult mice injected with a sheep red blood cell hemolysate supernatant fraction were found to have a severely reduced responsiveness to subsequent immunization with sheep red blood cells. The induced unresponsiveness was found to be specific, as tested by reciprocal experiments involving horse and sheep erythrocyte preparations. The tolerogenic material did not appear to be immunogenic.

The technical assistance of Phyllis Halloran and Louis Kubai in portions of this study is gratefully acknowledged.

**REFERENCES**

1. Friedman, H. 1965. Failure of spleen cells from immunologically tolerant mice to form antibody plaques to sheep erythrocytes in agar. Nature (Lond.). 206:508.
2. Friedman, H. 1969. Discussion. In Immunological Tolerance. M. Landy and W. Braun, editors. Academic Press, New York. 31.
3. Frish, A. W., and G. H. Davis. 1966. Inhibition of hemagglutinin synthesis by cytoxan: specificity and drug-induced tolerance. J. Lab. Clin. Med. 68:103.
4. Aisenberg, A. C. 1967. Studies on cyclophosphamide-induced tolerance to sheep erythrocytes. J. Exp. Med. 125:833.
5. Dietrich, M. F., and P. Dukor. 1967. The immune response to heterologous red cells in mice. III. Cyclophosphamide-induced tolerance to multiple species red cells. Pathol. Microbiol. 30:309.
6. Playfair, L. H. J. 1969. Specific tolerance to sheep erythrocytes in mouse bone marrow cells. Nature (Lond.). 222:882.
7. Schwartz, R. S. 1965. Immunosuppressive drugs. Prog. Allergy. 9:246.
8. Landy, M., and W. Braun, editors. 1969. Immunological Tolerance. Academic Press, New York.
9. Leskowitz, S. 1967. Tolerance. Annu. Rev. Microbiol. 21:157.
10. Weigle, W. O. 1971. Recent observations and concepts in immunological unresponsiveness and autoimmunity. Clin. Exp. Immunol. 9:437.
11. Palmer, J. 1972. Cited in Annual Report, Walter and Eliza Hall Institute of Medical Research, Melbourne, 1970–1971. G. J. V. Nossal, editor.
12. Fetherstonhaugh, P. 1970. The immunogenicity and tolerance-inducing ability of soluble extracts of sheep red blood cell membranes. Int. Arch. Allergy Appl. Immunol. 39:310.
13. Waterstone, H. R. 1970. Soluble antigen from sheep erythrocytes: preparation and antigenic properties. J. Immunol. 18:431.
14. Cunningham, J. A., and A. Szenberg. 1968. Further improvement in the plaque technique for detecting single antibody forming cells. J. Immunol. 14:599.
15. Anderson, T.-H. 1971. Tolerogenic effects of soluble erythrocyte antigens in neonatal and adult mice. Master's Degree Dissertation. University of Wisconsin, Madison.
16. Dresser, D. W. 1962. Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. Immunology. 5:378.