

TRANSFORMATION OF A HISTOCOMPATIBILITY IMMUNOGEN INTO A TOLEROGEN*

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Histocompatibility antigens provoke the rejection of allografts. They induce both humoral and cellular sensitivity. *H-2* antigens are the major histocompatibility antigens in mice and are analogous to the *H-LA* antigens in humans.

H-2 antigens are present on cell membranes. Both spleen cell membranes (SM)¹ and liver cell membranes (LM) from mice absorb alloantibody produced specifically against the *H-2* antigens of that strain of mice. However, SM and LM behave differently in vivo. Whereas SM causes accelerated skin-graft rejection and induces humoral antibody formation against its *H-2* antigens, LM has no effect on skin-graft rejection and induces humoral tolerance for its *H-2* antigens (1). There is evidence that SM contains seroantigens not present on LM (2). Thus, there are important differences between *H-2* antigens on cells of the two organs.

The following experiments explore these differences. The experiments show that mixing allogeneic SM with a syngeneic liver extract results in a preparation which absorbs antibody, which does not immunize for humoral antibody production or for accelerated skin-graft rejection, and which tolerizes for humoral antibody production; i.e., major properties of the allogeneic SM are transformed into those of allogeneic LM.

Materials and Methods

Mouse Strains.—The mouse strains used in these experiments were C57BL10Sn (*H-2^b*), C57BL10D2New/Sn (*H-2^d*), C57BL10Br (*H-2^k*), and C57B10A (*H-2^a*) provided by Jackson Laboratories, Bar Harbor, Me. These will be called *B*, *D*, *K*, and *A*, respectively. These strains are congenic with one another and thus presumably differ only at the *H-2* region of the mouse genome.

Membrane Preparation.—Membranes were prepared from spleen and liver by hypotonic salt extraction and differential centrifugation (3). Briefly, mice are killed by cervical dislocation and their organs immediately removed. The organs are then homogenized in 0.13 M NaCl (liver with a Teflon pestle and spleen with a glass grinder) and iced for 30 min. The homoge-

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¹ Abbreviations used in this paper: LM, liver cell membrane; MEM, minimal essential medium; PBS, phosphate-buffered saline; SM, spleen cell membrane.

nates are then spun at 2,000 rpm for 20 min; the supernatants are decanted and saved. The pellets are resuspended in 0.11 M NaCl, iced for 30 min, and spun again. The two supernatants are then pooled and spun at 100,000 *g* for 1 h. The resultant pellets are lyophilized as the final "membrane" products. By electron microscopy, these preparations contain pieces of membranes and endoplasmic reticulum with many microsomes but no nuclei and very few mitochondria.

Preparation of Liver-Spleen Mixture.—Fig. 1 depicts the method of preparation of the liver-spleen mixture used in these experiments. Fresh livers from *B* mice were homogenized and a pooled low-speed crude supernatant prepared exactly as in the methods for membrane prepa-

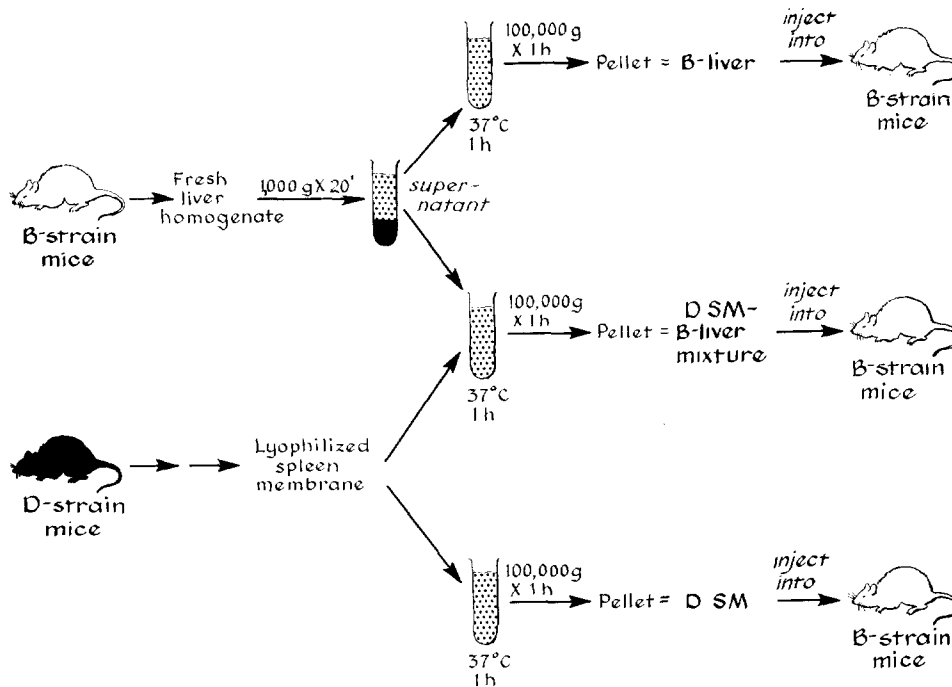


FIG. 1. Scheme of antigen preparation.

ration. This crude liver supernatant was then mixed with previously prepared SM from *D* mice at a ratio of supernatant from two livers for each 10 mg SM. The mixture was incubated at 37°C for 1 h and then spun at 100,000 *g* for 1 h. The resulting pellet was lyophilized as the final liver-spleen mixture, hereafter called mixture. Control preparations were made from identical amounts of liver incubated without SM, and SM incubated without liver. Because all liver and all recipient mice used in these experiments were *B* type, the liver was nonantigenic in this system; i.e., the only *H-2* antigens in the mixture recognizable by *B* recipients were those from the SM of *D* mice.

Skin Grafts.—Split thickness skin grafts 1 cm square were placed on the thorax of the recipient mouse and bandaged for 6 days. The grafts were scored grossly from the 6th day with 50% scab taken as the endpoint. Gross scoring for rejection is not as precise as microscopic scoring. However in these experiments the graft was needed for antigenic stimulation and could not be removed for sectioning. The process of scabbing was progressive and continued to complete scab formation. Previous comparisons of this scoring method with microscopic scoring

have shown the 50% scab endpoint to be consistent and reliable in distinguishing between first and second set responses.

Injections.—All injections were intraperitoneal. Lyophilized antigen (either the control preparation or the mixture) was suspended by homogenization in a volume of phosphate-buffered normal saline, pH 6.5 (PBS) so as to make the final volume of injection 0.2 ml per mouse. The precise amount of each antigen injected in lyophilized form and its equivalence in wet weight of tissue are given in the footnotes to the text. For injection of spleen cells, one fresh spleen was glass-ground in 3 ml PBS and 0.1 ml of the suspension injected per mouse.

Hemagglutination Antibody Assay (Hab Assay).—The titer of hemagglutinins in a serum was determined by the method of Herzenberg (4). The \log_{10} of the highest dilution of serum giving a 2+ reaction (0–4+ range) was taken as the “log titer” of a serum; the average of the “log titers” was calculated for each group and called the mean log titer.

Cytotoxic Antibody Assay (Cab Assay).—Cytotoxic antibody was assayed by measuring complement-dependent ^{51}Cr release from lymphocytes exposed to appropriate dilutions of serum (5). With the percent of ^{51}Cr release in distilled water set as 100% cytotoxicity, the dilution of antiserum which would produce 50% cytotoxicity was determined by interpolating or extrapolating from the dilutions tested. The \log_{10} of this titer was taken as the log titer of the serum. The mean log titer for each experimental group was calculated.

Antisera.—Reference *B* anti-*D* sera were prepared by injecting $\frac{1}{30}$ of a *B* spleen into *D* mice weekly for 6–12 wk. Other antisera came from various experimental groups as indicated in the text.

Absorptions.—To test lyophilized membrane preparations for absorbing activity, antisera were diluted 1:10 (in PBS for the Hab assay, in Earle's minimal essential medium [MEM] for the Cab assay) and 0.4 ml of this dilution was mixed with an aliquot of antigen in a homogenizing tube. The suspension was incubated for 30 min at 37°C and then spun for 10 min at full speed in an Adams serofuge (Clay-Adams, Inc., New York). The supernatant was removed and diluted appropriately for the assay.

Inhibitions.—As a more quantitative measure of antigenicity, a volume of various concentrations of antigen suspension was added to an equal volume of antiserum which had been diluted so as to give approximately 60–80% cytotoxicity in the Cab assay. The decrease in cytotoxicity in the presence of antigen was taken as a measurement of the antigenicity of the preparation.

Statistical Analysis.—Statistical significance between groups was determined by the Whitney-Mann U-Test. Data include standard error of the mean.

RESULTS

Initially, to test the antigenicity of the liver-spleen mixture, we performed simple absorptions of *B* anti-*D* serum. Preparations used for absorption were 1.4 mg *D* SM, 27.4 mg mixture, and 27.8 mg *B* liver.² Hemagglutination results using *D* cells are shown in Table I. Both the *D* SM and the mixture completely absorbed the hemagglutinating antibody. By contrast, a comparable amount of the nonantigenic *B* liver did not. In the latter absorption, as occurred about half the time, the residual titer was reduced. In view of the nonantigenic character of the absorbing material, the change presumably represents a nonspecific inter-

² 27.4 mg of mixture was the lyophilized yield from incubating 2.0 mg of lyophilized *D* SM with approximately 400 mg wet weight of *B* liver. 2.0 mg of *D* SM is the yield from approximately 100 mg of fresh spleen or one average spleen. 1.4 mg *D* SM and 27.8 mg *B* liver were the lyophilized yields from incubating separately 2.0 mg *D* SM or 400 mg wet weight of *B* liver. As is evident, some antigen is lost during the incubation and recovery.

ference with hemagglutination by the foreign material. As shown in Fig. 2, similar results were obtained with the cytotoxic assay using *D* lymphocytes. Again, both the *D* SM and the mixture fully absorbed the antiserum, while comparable amounts of *B* liver did not. Thus, as tested by absorption in either the hemagglutination or the cytotoxic assay, the mixture possessed antigenic activity similar to that of SM. On the other hand, the *B* liver being nonantigenic in this system did not. Different preparations of mixture always gave the same result.

As a more quantitative measure of antigenicity, the same preparations were used in an inhibition assay. Results are shown in Fig. 3. Serial dilutions of the antigen preparations were added to aliquots of the antiserum, and the test conducted as described under Methods. As before, *B* liver showed no antigenic activity, while *D* SM and the mixture showed significant activity. However, because a more dilute suspension of SM inhibited to the same degree as a more

TABLE I
Hemagglutinating Activity of B Anti-D Serum before and after Absorption by Test Antigens

Absorbing antigen	Reciprocal of Dilution of Serum							
	10	20	40	80	160	320	640	1,280
None	3	4	4	4	3	2	1	—
<i>D</i> SM	—	—	—	—	—	—	—	—
<i>B</i> liver	4	4	4	2	1	1	—	—
<i>D</i> SM- <i>B</i> liver Mixture	—	—	—	—	—	—	—	—

concentrated suspension of the mixture, the unreacted SM was more antigenic than a comparable amount in the mixture (i.e., an amount of mixture derived from the starting amount of SM). In fact, from Fig. 3 it can be determined that approximately a $\frac{1}{13}$ dilution of SM suspension would have inhibited to the point of 50% cytotoxicity, while approximately a $\frac{1}{6}$ dilution of mixture suspension would be needed for the same degree of inhibition. Hence, in this case SM was about twice as antigenic as the mixture.

Next, we tested the ability of the preparations to sensitize for accelerated skin-graft rejection. Recipient *B* mice received a single injection of antigen 4 days before receiving a *D* skin graft. Amounts used for each injection were 2 mg *D* SM, 40 mg *B* liver, and 40 mg of mixture.³ Table II shows the results. Grafts on untreated animals and on those animals receiving nonantigenic liver had the normal first set median survival time of 10 days. Mice receiving SM rejected their grafts in an accelerated fashion, i.e., 7 days. Finally, grafts on mice injected with the mixture were rejected in 10 days, a normal first set rejection.

³ These amounts were the lyophilized yields from incubating separately 3 mg *D* SM, 600 mg wet weight *B* liver, or a mixture of 3 mg *D* SM with 600 mg *B* liver. Slightly more material was used in these experiments than in the absorptions because we wished a final injection of *D* SM to equal the yield from approximately 100 mg wet weight of spleen. This amount of allogeneic spleen provokes a vigorous second-set graft rejection response to a subsequent skin graft.

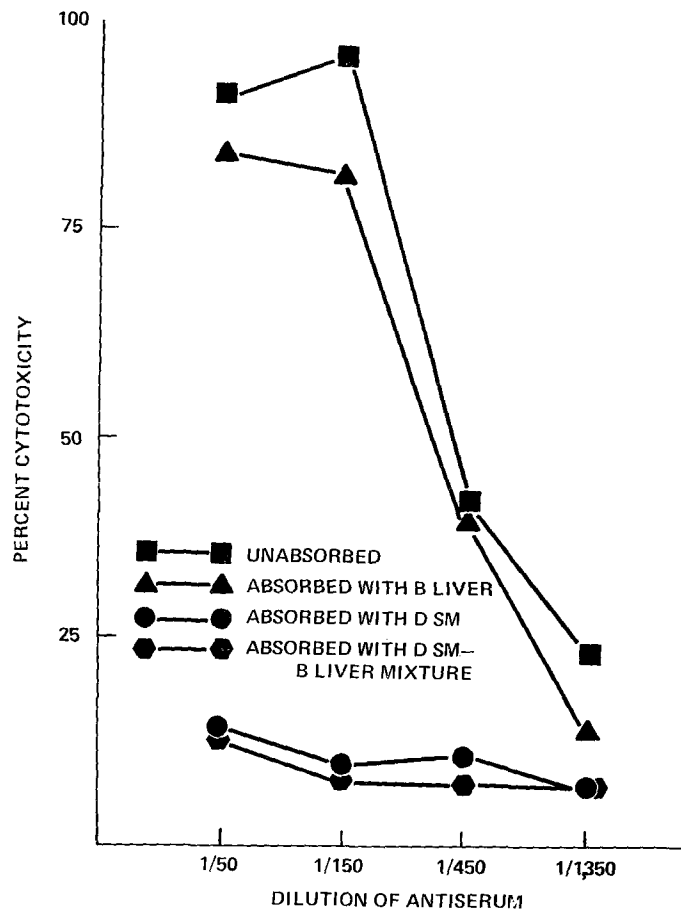


FIG. 2. Cytotoxic antibody of *B* anti-*D* serum before and after absorption by test antigen.

Thus, while SM sensitized for accelerated skin-graft rejection, the mixture did not.

The third column in Table II shows the cytotoxic antibody titers of the animals 13 days after grafting. All groups of animals had comparable antibody titers except those animals which had received the mixture. This group had significantly lower titers. Thus, one injection of the mixture induced humoral tolerance as measured by cytotoxic antibody production in response to a skin graft.

Additional experiments explored further the humoral response to these preparations. *B* mice received six weekly injections approximately of 0.2 mg *D* SM, 3.2 mg *B* liver, or 3.7 mg mixture.⁴ The mice were bled after this 6-wk pre-

⁴ These were the yields from incubation separately of 0.3 mg *D* SM, 60 mg wet weight *B* liver, or the mixture of 0.3 mg *D* SM, and 60 mg wet weight liver. The antigenic amounts in these experiments were designed to be the equivalent of approximately 10 mg wet weight of spleen per injection, which had been found to provoke good humoral sensitization in this type of multiple injection experiment.

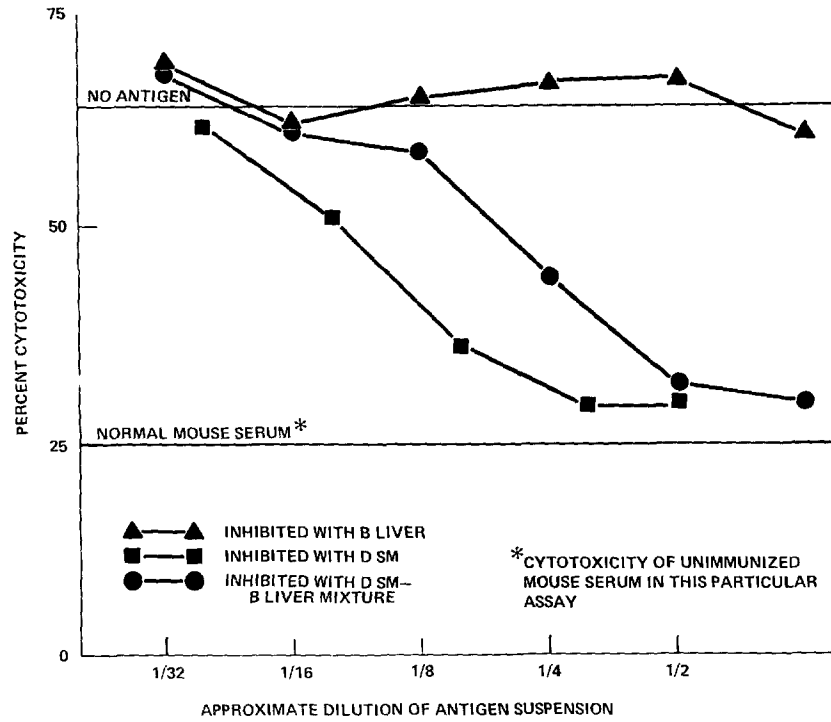


FIG. 3. Quantitative inhibitions of *B* anti-*D* serum by test antigen.

TABLE II

Responses of B Mice Pretreated with One Injection of Test Antigen 4 Days before Receiving D Skin Graft

Group*	Test antigen used in pretreatment	Median skin graft survival time	Mean log cytotoxic antibody titer 13 days after grafting
		<i>days</i>	
1	None	10 ± 0.7	2.42 ± 0.14
2	<i>B</i> liver	10 ± 0.4	2.54 ± 0.18
3	<i>D</i> SM	7 ± 0.1†	2.71 ± 0.16
4	<i>D</i> SM- <i>B</i> liver mixture	10 ± 0.6	1.62 ± 0.22†

* Number of animals in each group: 1 = 14, 2 = 15, 3 = 20, and 4 = 16.

† $P \leq 0.01$.

treatment and then challenged with weekly injections of cells from $\frac{1}{30}$ of a *D* spleen. Cytotoxic assay results are shown in Fig. 4. Animals receiving six weekly injections of *D* SM developed significant antibody titer. However, those mice receiving six injections of the mixture did not. As expected, a control group which received weekly injections of syngeneic liver developed no antibody. Thus, the

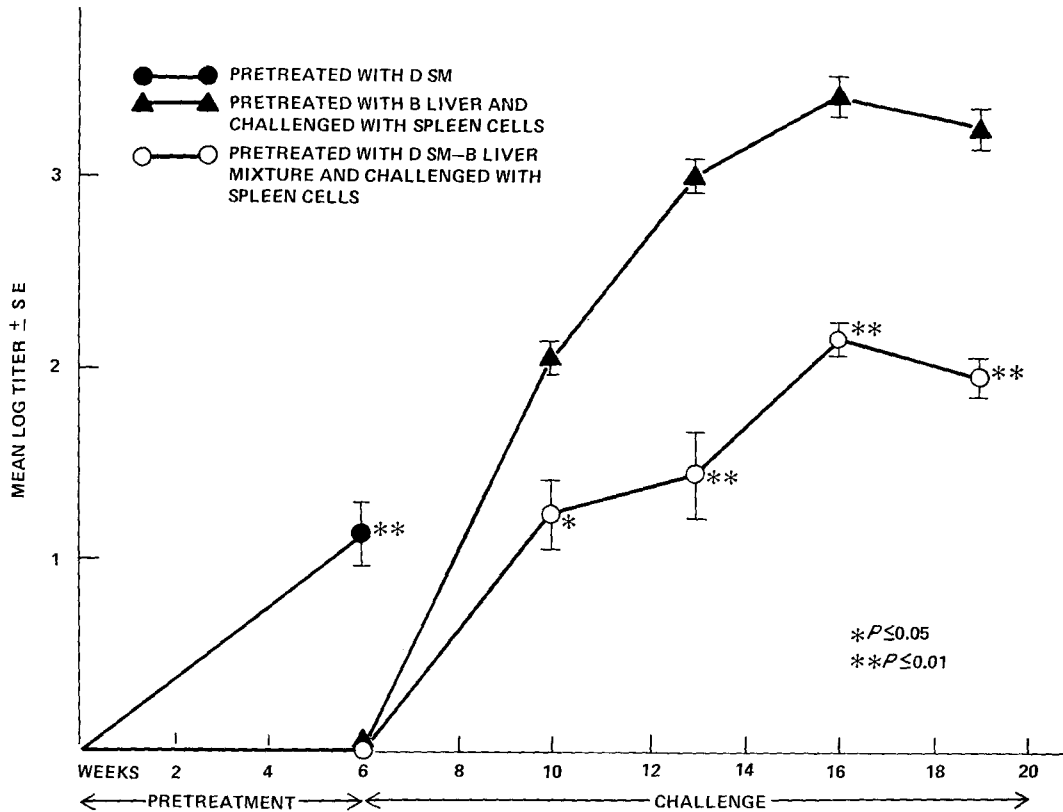


FIG. 4. Tolerogenicity of altered SM for cytotoxic antibody formation. 11 animals in the group treated with SM. 12 animals in the other two groups.

SM was immunogenic and the mixture was not. Furthermore, upon subsequent challenge by weekly injections of *D* spleen cells, the mice pretreated with mixture developed significantly lower titers than the controls injected with syngeneic liver. Not shown on this graph, unpretreated controls also challenged with *D* spleen developed titers similar to those of the controls pretreated with syngeneic liver. This result represented a greater than 90% decrease in amount of antibody formed in mice treated with mixture as compared to controls.

Fig. 5 shows the hemagglutinating antibody titers of the same mice. Again, *D* SM was immunogenic while the mixture was not, and the mixture was tolerogenic compared to controls.

Thus, as measured by either cytotoxic or hemagglutinating antibody, allogeneic *D* SM was immunogenic for humoral antibody production, while the allogeneic SM pretreated with liver was tolerogenic.

Finally, the tolerance induced by the mixture was shown to be immunologically specific. Groups of *B* mice were injected weekly either with 4 mg of the mixture

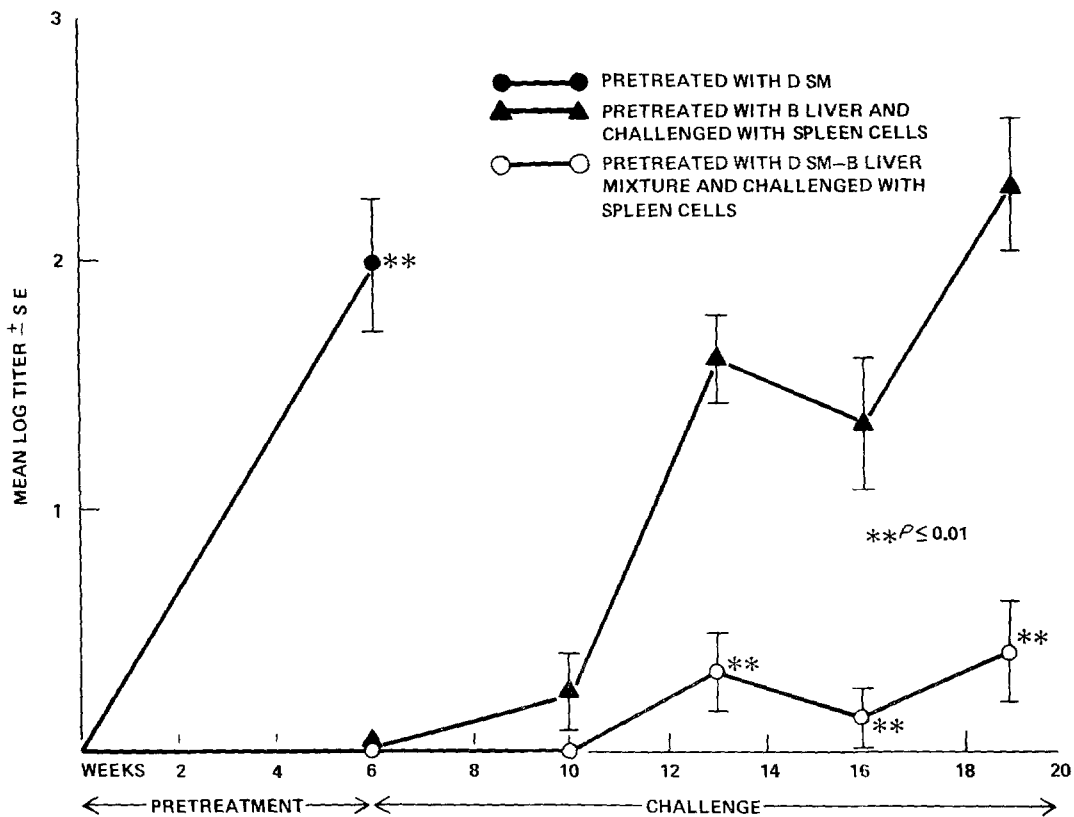


FIG. 5. Tolerogenicity of altered SM for hemagglutinating antibody formation. Same animals as in Fig. 4.

or with 7 mg of *B* liver along.⁵ After 6 wk, the animals were bled, and both of the above groups were divided randomly into two subgroups—one subgroup to be challenged with *D* spleen cells and one with *A* spleen cells. The design of this experiment is based in part upon the fact that *A* seroantigens are predominantly a combination of those of *D* and *K*. The results from those animals challenged with *D* spleen cells are shown in the first column of Table III. The animals injected with the mixture and challenged with *D* spleen cells were tolerant when compared with animals pretreated with *B* liver and similarly challenged.

The results from those mice challenged with *A* spleen cells are shown in the remaining columns of Table III. The second column shows that sera from animals treated with mixture reacted well with *A* cells, i.e., the titers were not significantly different from those of control sera. However, the third column shows

⁵ Amounts recovered from incubation of 0.3 mg *D* SM plus 60 mg wet weight *B* liver and from 60 mg wet weight *B* liver alone, respectively.

TABLE III
Serum Cytotoxic Antibody Mean Log Titers of B Mice Pretreated for 6 wk as Indicated and then Challenged with 12 Weekly Injections of Spleen Cells

Pretreatment	Challenged w/D spleen		Challenged w/A spleen	
	Assayed with D lymphocytes	Assayed with A lymphocytes	Assayed with D lymphocytes	Assayed with K lymphocytes
B liver	1.65 ± 0.14	1.93 ± 0.20	1.29 ± 0.22	1.25 ± 0.13
D SM-B liver mixture	1.03 ± 0.13*	1.61 ± 0.14‡	0.71 ± 0.07§	1.04 ± 0.05‡

Eight animals in each group. *H-2* seroantigens present on above lymphocytes which are recognized by *B* mice are: *A* = 1, 3, 4, 8, 10, 11, 13, 25; *D* = 3, 4, 8, 10, 13, 31; *K* = 1, 3, 8, 11, 25, 32.

* $P \leq 0.01$.

‡, insignificant difference.

§ $P \leq 0.05$.

that these same sera from animals treated with mixture reacted with *D* cells at a level significantly lower than did sera from controls. As shown in the last column, these sera reacted with *K* cells at levels similar to control sera. *K* antigens constitute essentially those *A* antigenic specificities not present on *D* cells. Thus, mice treated with mixture and challenged with *D* cells were tolerant and those challenged with *A* cells showed significant tolerance to *D* antigens, but not to the other *A* antigens. Hence, the tolerance was specific.

DISCUSSION

In previous work on histocompatibility antigens by Mandel et al. (6) and by Palm and Manson (7), as in the present work, incubation with liver was found to eliminate the ability of spleen to sensitize for accelerated skin graft rejection. This was interpreted to indicate that liver destroyed spleen histocompatibility antigens. Similarly, Hilgert and Kristofova (8) found that in some systems pretreatment with liver eliminated the ability of spleen to immunize for antibody production as well as to sensitize for accelerated skin graft rejection. However the induction of humoral tolerance was not studied. Furthermore other syngeneic tissues had the same effect as liver. These results were attributed to either a destruction of spleen antigens or to a nonspecific inhibitory effect upon immunization of the added tissue in the inoculum. We have now shown that liver extract does not destroy the spleen humoral antigenicity nor does it nonspecifically inhibit immunogenicity. Instead, it transforms the spleen membrane *H-2* antigen from an immunogen to a tolerogen.

Precedent for transformation of an immunogen into a tolerogen was established in experiments with flagellin by Parish and others (9). A physical alteration, i.e., cyanogen bromide cleavage, created a tolerogenic product from immunogenic flagellin. In our experiments liver may be inducing a physical change in the spleen antigens, possibly enzymatically, thereby changing the in vivo

properties of the SM from humoral immunogenicity to tolerogenicity. For example, a liver enzyme might be destroying or cleaving a part of the SM antigen which is necessary for immunization. Support for this possibility derives from the fact that during incubation of the *D* SM and *B* liver, soluble *D* antigen is released into the supernatant, indicating that at least some physical alteration is occurring. Alternatively, liver may be uncovering an adjacent membrane portion which combines with antigen to produce a tolerogen. It is also possible that the tolerance is not a result of an initial liver-spleen interaction but rather is a result of separate effects of liver and spleen on the animal occurring only after injection. However we have found no evidence of tolerance when liver homogenate and *D* SM were injected separately without prior incubation together.

Another hypothetical alternative explanation to transformation of the antigen would be a form of "low dose" tolerance. This would postulate destruction of most but not all SM by the liver homogenate; the small amount of remaining SM would induce a "low dose" tolerance. This is almost certainly not the case. First, the mixture absorbs antibody almost as well as SM alone indicating that most if not all antigenicity remains. Second, a single dose of mixture is tolerogenic in the graft rejection experiments; to our knowledge "low dose" tolerance has not been produced by single doses. Third, we have experimented with multiple injections of SM in doses varying from 0.01 to 1.0 mg per injection. No evidence of low dose humoral tolerance was found.

Solubilization of histocompatibility antigens from cell membranes has been achieved by autodigestion, by treatment with KCl and by digestion with papain (10). These procedures leave a residue of varying antigenicity. In preliminary experiments we have found the residue from sequential treatment of SM with papain and KCl to be highly immunogenic for antibody to *H-2* antigens. Thus, despite the fact some solubilization of antigen occurs in each case, the action of liver homogenate on SM appears to differ from that of KCl and papain.

Whatever the action of the liver, because both the liver and the recipient mice were always of the *B* strain, *H-2* antigenicity of the liver is not involved. As yet, we cannot identify the active component of the liver homogenate or the alteration which it causes. Study of this system may allow further delineation of the structure of histocompatibility antigens and the differences between their immunogenic and tolerogenic forms.

Although not affecting cellular sensitivity, a humoral histocompatibility tolerogen might find clinical application in preventing those components of rejection of grafted organs which are caused by humoral antibody, and in preventing rejection of free cell grafts, such as platelets and white cells, where humoral antibody presumably has a large role in rejection.

Finally, the alteration of spleen histocompatibility antigen by a constituent of liver raises a theoretical point regarding gene expression. Although all cells of an animal contain the same histocompatibility genes, histocompatibility antigens are represented differently on different organs. One explanation, among

several, is synthesis of different gene products in the different tissues. An alternative hypothesis, raised here, is alteration of the already formed gene product by different intracellular enzymes, which are not necessarily controlled by the histocompatibility genes.

SUMMARY

H-2 antigens on spleen cell membranes absorb antibody to *H-2* antigens and induce both humoral and cellular responses. Liver cell membrane *H-2* antigens by contrast also absorb antibody but do not influence cellular response and are tolerogenic for the humoral response. This paper demonstrates that syngeneic liver cells contain a substance which can transform the properties of allogeneic spleen cell membranes into those of allogeneic liver cell membranes, i.e., transform a humoral immunogen into a humoral tolerogen. The process appears to be accompanied by cleavage of an antigen component from the spleen membrane and hence to result in a structural change in the *H-2* antigen.

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REFERENCES

1. Holman, H. R., R. Uebbing, and W. Davis. 1967. Induction of simultaneous immunological paralysis and sensitization to different antigenic activities controlled by the major histocompatibility locus in the adult mouse. *Trans. Assoc. Am. Physicians. Phila.* **80**:142.
2. Hotti, A., S. Fu, and H. Holman. 1970. Variable representation and immunogenicity of histocompatibility antigens on different tissues. *J. Clin. Invest.* **49**:46a. (Abstr.)
3. Mann, D. L., G. Rogenting, Jr., J. L. Fahey, and S. G. Nathenson. 1968. Solubilization of human leucocyte membrane isoantigens. *Nature (Lond.)*. **217**:1180.
4. Herzenberg, L., and L. Herzenberg. 1961. Association of H-2 antigens with the cell membrane fraction of mouse liver. *Proc. Natl. Acad. Sci. U. S. A.* **47**:762.
5. Wigzell, H. 1965. Quantitative titrations of mouse H-2 antibodies using Cr⁵¹-labelled target cells. *Transplantation*. **3**:423.
6. Mandel, M. A., A. P. Monaco, and P. S. Russell. 1965. Destruction of splenic transplantation antigens by a factor present in liver. *J. Immunol.* **95**:673.
7. Palm, J., and L. A. Manson. 1965. Tissue distribution and intracellular sites of some mouse isoantigens. In *Isoantigens and Cell Interactions*. J. Palm, editor. Wistar Institute Press, Philadelphia.
8. Hilgert, I., and H. Kristofova. 1967. Immunogenic properties of cell-free tissue extracts in mice. *Transplantation*. **5**:1256.
9. Parish, C. R., and G. L. Ada. 1969. The tolerance inducing properties in rats of bacterial flagellin cleaved at the methionine residues. *Immunology*. **17**:153.
10. Reisfeld, R. A., and B. D. Kahan. 1970. Transplantation antigens. *Adv. Immunol.* **12**:117.