T-CELL-DEFICIENT MICE PRODUCE MORE ANTIHAPTEN ANTIBODIES AGAINST SYNGENEIC THAN AGAINST ALLOGENEIC ERYTHROCYTE CONJUGATES*

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In cell interactions involving lymphocytes histocompatibility is often a requirement for success both in collaborative and aggressive interactions. Such interactions include the helper function of T lymphocytes in the activation of B lymphocytes. Earlier evidence suggested that the T and the B lymphocyte must share alleles of the *H-2* locus (1–7). Later studies speak against such a requirement (8–11), but *H-2* compatibility may be required between cell types that interact in the generation of helper cells.

Histocompatibility was also required for an optimal interaction of lymphocytes and antigen-pulsed macrophages leading to proliferation of the lymphocytes (12, 13). Finally, it was found necessary for the generation of cytotoxic T lymphocytes and their killing function in several conditions. These conditions include some viral infections (14–17), in vitro immunization by hapten-coupled spleen cells (18, 19), and in vivo immunization by allogeneic cells (20) or male cells (21).

Some of the above studies suggested that lymphocytes could recognize self-structures (probably products of the *H-2* chromosomal region) on other cells, and this recognition enhanced triggering of the lymphocytes. This raised the possibility that a hapten coupled to syngeneic cells might be a stronger immunogen than the same hapten coupled to allogeneic cells. Our earlier studies did not support this hypothesis (22, 23), but this could have been due to competition of two enhancing factors. The response to syngeneic conjugates perhaps was enhanced by self-recognition, but the response to allogeneic cells might have been enhanced by recognition of alloantigens by helper T cells. Therefore we decided to compare immunogenicity of haptenated syngeneic and allogeneic erythrocytes in T-lymphocyte-deficient mice.

Materials and Methods

Animals. 3–4-mo-old female mice of strains CBA ($H\text{-}2^k$), C3H ($H\text{-}2^k$), C57BL/6 ($H\text{-}2^b$), LP/J ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$), B10Br ($H\text{-}2^k$), B10D2 ($H\text{-}2^d$), and F_1 (CBA × C57BL/6) were used. They were bred in this laboratory. B10Br and B10D2 are congenic strains with C57BL/10Sn background genome. C57BL/10Sn strain greatly resembles the C57BL/6 strain. 6–8-wk-old nude (*nu/nu*) mice and their heterozygous *nu*/+ littermates were also from our own colony. They had mostly C57BL/6 background genome, at least three backcrosses to the C57BL/6 were in their immediate pedigree.

* This study was supported by the Medical Research Council, Academy of Finland.

Antigens and Immunization. NNP[1] (4-hydroxy-3,5-dinitrophenyl acetic acid), NNP-azide, and NNP-ovalbumin (OA) conjugates were prepared according to Brownstone et al. (24). In the conjugation of NNP-azide to mouse red blood cells (MRBC) a method of Pasanen and Mäkelä (25) with slight modifications (23) was employed. Briefly, MRBCs were taken to Alsever's solution and washed three times with normal saline. A 20% suspension was made in 0.12 M carbonate-bicarbonate buffer (CBB) pH 9.25. A 4% solution of NNP-azide in *N-N*-dimethylformamide was diluted 10-fold with the CBB. Equal volumes of this solution and 20% erythrocytes were mixed and allowed to stand at room temperature for 1 hr. The cells were washed four times with saline. NNP-OA containing 5 M of NNP per 1 mol of OA was coupled to red cells according to the method described by Vyas et al. (26). 1 ml of three times washed packed red cells were added to a mixture containing 10 ml of NNP-OA solution (6 mg/ml in saline) and 10 ml $CrCl_3$ solution prepared from 1% stock solution by dilution 1:100 in saline immediately before use. The mixture was allowed to stand at room temperature for 5 min with constant gentle stirring and then centrifuged. The cells were washed four times with saline and tested with rabbit anti-NNP serum with hemagglutination. Cells that gave the hemagglutination titer 1/128–1/256 were accepted. All conjugates were prepared on the day they were used. In immunization 0.2 ml of 25% coupled MRBC suspension (10^9 cells) in saline was given intraperitoneally.

Anti-NNP plaque-forming cell (PFC) response was assayed on day 4 or 5. Only direct PFC were determined in most experiments.

Anti-Thymocyte Serum, ATS. ATS was rabbit anti-mouse thymocyte serum from Microbiological Associates Inc., Bethesda, Md. (lots 13033 and 15038). They were absorbed with 1/3 vol of MRBC for 1 h at +4°C. 0.3 ml was injected intraperitoneally simultaneously with the antigen from a separate syringe.

Hemolytic Plaque Assay. The number of PFC was assayed with NNP-coupled sheep erythrocytes which were prepared for each assay in the same way as the MRBC conjugate, but the NNP-azide concentration of the final conjugation mixture was 0.1 mg/ml. The guinea pig complement was absorbed at +0°C by incubating the undiluted serum (10 vol) with packed NNP-MRBC (1 vol) for 1 hr. The plaque technique was a modification of that developed by Cunningham and Szenberg (27). Briefly, 0.3 ml of 10% NNP-SRBC suspension and 0.1 ml of undiluted guinea pig serum were added to 0.6 ml of RPMI 1640 medium. A 0.4-ml aliquot of this mixture was mixed with 0.2 ml of the appropriate spleen cell suspension, and aliquots of this mixture were pipetted into slide chambers calibrated to have a capacity of 0.1 ml. The chambers were sealed with paraffin and incubated for 30 min at 37°C. Plaques were then counted under a dissection microscope. Uncoupled SRBC were used as control. For each assay three chambers were used.

Results

The response of T-Cell-Deficient Mice to NNP Conjugates of Syngeneic, Allogeneic, or Xenogeneic Erythrocytes. Four types of T-cell-deficient mice, ATS-treated CBA, C57BL/6, F_1, or nude mice with predominantly C57BL/6 background, were tested. They all mounted a significantly stronger anti-NNP response to NNP-conjugates of syngeneic erythrocytes than against conjugates of allogeneic or xenogeneic erythrocytes (Table I, Column I, II, and V). In preliminary experiments both direct and indirect PFCs were determined. Few indirect PFCs were found, and only the values for direct PFCs are reported.

In some experiments the donors of the NNP-MRBC shared the *H-2* genes with the recipient strain, but were otherwise allogeneic. The responses were nearly as high as the responses to syngeneic erythrocytes (Column III). Two groups of C57BL/6 mice were also tested with *H-2* incompatible but otherwise similar erythrocytes (B10/Br or B10/D2), and the response was as weak as it was against completely allogeneic or xenogeneic conjugates (Table I, Column IV).

[1] *Abbreviations used in this paper:* ATS, anti-thymocyte serum; CBB, carbonate-bicarbonate buffer; MRBC, mouse red blood cell; NNP, 4-hydroxy-3,5-dinitrophenyl acetic acid; OA, ovalbumin; PFC, plaque-forming cell; SRBC, sheep red blood cells.

TABLE I

The Anti-NNP-PFC Response (PFC per 10^6 Spleen Cells) to Syngeneic, Allogeneic, or Xenogeneic NNP-Erythrocyte Conjugates in T-Cell-Deficient Mice

Recipient	Fully syngeneic donor	Allogeneic donor	*H-2* compatible but allogeneic donor	*H-2* incompatible but otherwise very similar donor	Xenogeneic donor (SRBC)
	I	II	III	IV	V
ATS-CBA	478 (1.12)	248 (1.12)	435 (1.17)		
ATS-C57BL/6	427 (1.12)	137 (1.20)	303 (1.12)	137 (1.17)	137 (1.12)
C57BL/6 *nu/nu*	448 (1.17)	174 (1.14)			
ATS-F_1 (CBA × C57BL/6)	389 (1.09)	146 (1.07)			

Mice were immunized i.p. with 0.2 ml of 25% NNP-MRBC. Non-nude mice received a simultaneous injection of ATS (0.3 ml). Nude mice had mainly C57BL/6 background. The donor strains in column IV were B10Br and B10D2. Donor strains for column III were B10Br (for CBA) and LP/J (for C57BL/6). Direct anti-NNP-PFC on day 4 are given (geometric means). Numbers in parenthesis indicate the coefficient of variation. It is equivalent to sign x/÷ and is analogous for log normal distribution to standard error. One to five experiments were pooled for each number. Each recipient strain produced significantly more anti-NNP-PFC in response to syngeneic than allogeneic, xenogeneic, or "only *H-2* incompatible" conjugates ($P < 0.005$). Each recipient strain produced significantly more anti-NNP-PFC in response to allogeneic but *H-2* compatible than against allogeneic, xenogeneic, or "only *H-2* incompatible" conjugates ($P < 0.01$). The number of recipients tested was 8–10 for each value except the means CBA I and II (it was 24), C57BL/6 I (it was 15), C57BL/6 *nu/nu* II (it was 4), and C57BL/6 V (it was 5). PFC/10^6 spleen cells against SRBC were 7.6, x/÷ 1.17. ATS, ATS treated.

The next step was to compare the semiallogeneic combination (F_1 hybrid vs. parental) with the allogeneic and syngeneic combinations. Data in Table II show that if parental erythrocytes were injected into F_1 hybrids the response was intermediate, significantly lower than the response to syngeneic conjugates ($P < 0.025$) but higher than the response to allogeneic conjugates ($P < 0.005$). F_1 hybrid erythrocyte conjugates injected into parental recipients induced responses that were as weak as responses to allogeneic erythrocyte conjugates (Table II).

The Response of Normal Mice to NNP Conjugates of Syngeneic, Allogeneic, or Xenogeneic Erythrocytes. The response of normal mice to NNP conjugates of syngeneic cells was weaker than the response of T-cell deficient mice (Table III). CBA cells into C57BL/6 also resulted in a low response, but the other combination (C57BL/6 cells into CBA) induced a two to three times stronger response than did syngeneic CBA conjugates.

Mice of the C57BL/6 strain were also immunized with NNP-SRBC. The response of normal mice was 12 times higher than their response to syngeneic or allogeneic NNP conjugates, but the response of ATS-treated mice was the same as their response to allogeneic conjugates and two and one-half times lower than the response to syngeneic conjugates (Table III). These data suggest that the anti-NNP response to NNP-SRBC was strong when T-cell help was available, but it was as weak as the response to allogeneic NNP-MRBC without such help.

The Response of T-Cell-Deficient Mice to NNP-OA Conjugates of Syngeneic and Allogeneic Erythrocytes. Results reported above suggested that an optimal anti-NNP response to NNP-MRBC conjugates in T-cell-deficient mice was

TABLE II

The Anti-NNP-PFC Response (PFC per 10^6 Spleen Cells) in ATS-Treated Parental and F_1(CBA × C57BL/6) Mice Immunized Either with NNP-Conjugated Parental, Allogeneic, or F_1 (CBA × C57BL/6) Erythrocytes

Recipient/Donor	F_1 (CBA × C57BL/6)	CBA	C57BL/6	BALB/c
F_1(CBA × C57BL/6)	389 (1.09)	286 (1.07)	273 (1.07)*	146 (1.07)
CBA	152 (1.20)	699 (1.12)	226 (1.17)	
C57BL/6	87 (1.09)	137 (1.20)	475 (1.09)	

Each value is a pool of two experiments and 10 mice if not otherwise indicated. Recipients for syngeneic and allogeneic MRBC conjugates are included in the results of Table I. For other explanations see Table I. The first value on the first line is significantly higher than the second and the third ($P < 0.025$ or 0.005), which again are significantly higher than the fourth ($P < 0.005$). On the two other lines the syngeneic combination (underlined) produced significantly higher values than the other combinations ($P < 0.005$). Other comparisons exhibit no statistically significant differences ($P > 0.05$).

* 20 mice, three experiments.

TABLE III

The Anti-NNP-PFC Response (PFC per 10^6 Spleen Cells) in Normal and T-Cell-Deficient Mice Immunized with Syngeneic or Allogeneic NNP-MRBC

Recipient	Syngeneic donor	Allogeneic donor	Xenogeneic donor	No immunization
CBA normal	207 (1.23)	484 (1.34)		52 (1.38)
ATS-treated	446 (1.14)	294 (1.34)		65 (1.12)
C57BL/6 normal	162 (1.07)	167 (1.12)	2,096 (1.12)	25 (1.12)
ATS or *nu/nu*	424 (1.12)	126 (1.17)	137 (1.12)	32 (1.17)

Each value is a pool of three to four experiments where one NNP-MRBC batch was given to both normal and T-cell-deficient mice of both strains, except the values for xenogeneic donor cells (0.2 ml of 25% SRBC) derived from one experiment. The results of T-cell-depleted mice are included in the results of Table I. The number of recipients per each mean varied from 9 to 12 except in the groups of xenogeneic donor and nonimmunized mice it was 5. Statistical significances are discussed in the text. For other explanations see Table I.

obtained when antigenic recognition and self-recognition occurred together. It is of interest whether these two recognitions were performed by one combining site as appears to have been the case in the experiments of Zinkernagel and Doherty (16, 17) and Shearer et al. (19, 28) and Forman (29) and Tokuyama (30). The other possibility was that the antigenic recognition and self-recognition were two independent events. Trying to decide between these alternatives we made the NNP hapten a part of a constant foreign structure OA and conjugated this complex to either syngeneic or allogeneic erythrocytes. ATS-treated CBA and C57BL/6 mice were immunized both with CBA and C57BL/6 erythrocytes coupled with NNP-OA. The results in Table IV indicate that despite of the separation of the NNP and self-structures, syngeneic conjugates induced a stronger anti-NNP response than allogeneic conjugates speaking for the latter alternative.

TABLE IV
Anti-NNP-PVC/10^6 Spleen Cells in T-Cell-Deficient Mice Immunized with Syngeneic or Allogeneic NNP-OA-MRBC

Recipient	No. of mice per group	Syngeneic conjugate	Allogeneic conjugate	*P*
C57BL/6 Exp. 1a	8	254	75	<0.005
Exp. 2a*	4	320	168	<0.025
Exp. 3a	4	314	120	<0.005
Total	16	284 ×÷ 1.07	104 ×÷ 1.12	<0.005
CBA Exp. 1b	8	351	184	<0.025
Exp. 2b	4	365	190	<0.025
Exp. 3b	4	410	144	<0.005
Total	16	370 ×÷ 1.07 175 ×÷ 1.09	<0.005	
Total	32	324 ×÷ 1.04	134 ×÷ 1.07	<0.005

Direct anti-NNP PFC on day 5 are given. Syngeneic conjugate in experiment a served as allogeneic conjugate in experiment b and vice versa. These mice contained the average of 8.4 anti-SRBC per 10^6 spleen cells. C57BL/6 mice immunized with NNP-OA-SRBC had the average of 92 PFU per 10^6 when tested with NNP-SRBC. For other explanations see Table I.

* Recipients were nude mice with C57BL/6 background.

Discussion

T-cell-deficient mice produced two to three times more hapten-specific PFC when immunized with syngeneic erythrocyte conjugates of the hapten than when immunized with allogeneic or xenogeneic conjugates. Critical for this effect was identity in *H-2* of the responder cells and antigenic cells. Complete or nearly complete enhancement of the response was obtained with haptenated allogeneic erythrocytes that shared the *H-2* characteristics with the responder mice. On the other hand, if the *H-2* locus was dissimilar, almost complete identity in the background genomes did not result in any increase in the hapten-specific PFC.

Our finding adds an entry into the list of cellular interactions which are enhanced by identity or similarity in the *H-2* locus of the partner cells: interaction of B lymphocytes specific for a hapten and erythrocytes carrying this hapten on their surface (the antigen). This interaction led to activation of the B lymphocytes. Since this response was stronger in nude mice than in their normal littermates it was probably a T-cell independent response. Probably an interaction between B lymphocytes and haptenated erythrocytes was sufficient for the triggering of the lymphocytes. If the erythrocytes and the lymphocytes shared *H-2* structures it probably helped the triggering.

For the generation of cytotoxic lymphocytes slightly modified syngeneic cells have usually been used. They have been chemically modified (19, 28, 29), modified by a virus (14–17), or carrying a non-H alloantigen (20). In one case (31) they were probably fully syngeneic, and the cytotoxic lymphocytes probably belonged to "forbidden clones" (32) that recognized self-antigens.

In many of the earlier cases receptor antibodies of lymphocytes seem to have recognized an entity that has been called a "modified self structure." Another possibility has not been excluded, however, that a conventional antigen-recognition and self-recognition were two separate functions of the same responding lymphocyte. In the phenomenon that we report antigenic recognition and self-recognition probably were two independent events. It is unlikely that the combining site of a receptor antibody could have accommodated both the OA-conjugated NNP and the self-structure in the experiments of Table IV. Another fact against the first alternative is that we did not detect any self-preference in the effector cells, the PFC. In unpublished experiments we tested immune spleen cells with syngeneic or allogeneic NNP-MRBC. If alternative I were correct we expected a higher number of PFC with syngeneic than with allogeneic target cells when the mouse was immunized with syngeneic NNP-MRBC (17). No systematic preference was observed.

In many of the listed situations a semiallogeneic combination (F_1 vs. parental) was similar to a syngeneic combination. This was not a universal truth, however. In the activation of T lymphocytes by antigen-coated macrophages the semiallogeneic combination resulted to a considerably weaker stimulation than a syngeneic combination but to a stronger stimulation than an allogeneic combination (12, 13). In the cytotoxicity of autosensitized lymphocytes (31) F_1 hybrid targets generated cytotoxic lymphocytes, but these reacted only with parental cells, not with the heterozygous cells.

In our case the semiallogeneic combination resulted in an intermediate immunization if the antigenic cells came from either one of the parental strains, and the recipient animals were F_1 hybrids. The reciprocal combination (F_1 hybrid NNP-erythrocytes to a parental recipient) yielded weak responses that were indistinguishable from the responses in allogeneic combinations. A possible explanation for the weak response elicited by F_1 hybrid erythrocytes is that the amount of self-recognition structures was too low in heterozygous erythrocytes. It may be supoptimal even in homozygous erythrocytes, erythrocytes have little H-2 antigens (33–36), but at least it is likely to be twice as high as in heterozygous erythrocytes. It is more difficult to explain why the response by F_1 lymphocytes to parental erythrocytes was lower than to F_1 erythrocytes (but higher than the response to allogeneic erythrocytes). By this argument we might have expected the response of F_1 mice to parental erythrocytes to be as high as to F_1 erythrocytes. That this was not completely true may be due to a bad match between the different cell surfaces.

One might argue that the higher response to syngeneic than nonsyngeneic erythrocytes is due to a shorter survival of nonsyngeneic than syngeneic erythrocytes in the circulation. This is unplausible since erythrocytes conjugated with the NNP-azide concentration we used or even with one-tenth of it (0.2 mg/ml) disappear rapidly from the circulation (23). It is made even less plausible by the finding that parental erythrocytes in F_1 hybrid mice caused a submaximal response. No isoantibodies can clear them from circulation.

Another possible explanation for the lower anti-NNP response to allogeneic than syngeneic conjugates is allogeneic competition. The competing antigens would then be NNP and the alloantigens. We have unpublished evidence

against this possibility. A limited number of A/J mice were immunized with NNP conjugates of A/J, CBA (only the *K* end of *H-2* is shared with A/J), BALB/c (only *D* end of *H-2* is shared with A/J), or C57BL/6 erythrocytes. In these experiments identity in either end of the *H-2* complex was enough to make conjugates behave like syngeneic conjugates. This was also true of A/J erythrocyte conjugates injected to either BALB/c or CBA mice.

CBA mice which had a normal T-cell population exhibited a reverse picture, the response to allogeneic conjugates was stronger than to syngeneic conjugates. Since the response to allogeneic conjugates was strongly reduced by ATS treatment (which increased the response to syngeneic conjugates) the response to allogeneic conjugates was probably enhanced by alloreactive helper cells. This phenomenon could not be demonstrated in C57BL/6 mice immunized with CBA erythrocyte conjugates. Experiments are in progress that may tell us whether the C57BL/6 mice can respond to erythrocyte conjugates of other mouse strains.

The third conclusion that can be drawn from our experiments is that T-cell-deficient mice respond more strongly than normal mice to conjugates of syngeneic erythrocytes. This finding confirmed an earlier finding of Naor et al. (37). Both series of experiments suggest that thymus-derived cells act as suppressor cells in the anti-hapten response to conjugates of syngeneic erythrocytes. It is possible that they act as suppressor cells also in responses to allogeneic conjugates, but then the T-cell helper effect is stronger than the suppressor effect.

Summary

T-cell-deficient mice, either anti-thymocyte serum treated or nude mice, were immunized with hapten (4-hydroxy-3,5-dinitrophenyl acetic acid, NNP) conjugates of syngeneic, allogeneic, or xenogeneic erythrocytes. Immunization with syngeneic conjugates led to a stronger anti-NNP response than immunization with allogeneic or xenogeneic conjugates. A study of congenic mouse strains suggested that a prerequisite for this effect was that immunogenic erythrocytes and responding animals shared *H-2*-controlled characteristics.

F_1 hybrid erythrocyte conjugates injected into F_1 hybrid mice behaved like other syngeneic erythrocytes. The same erythrocyte conjugates injected into either parental strain induced a weak response indistinguishable from the response to allogeneic erythrocyte conjugates. Parental erythrocyte conjugates injected into F_1 mice induced an anti-NNP response that was significantly lower than the response to F_1 erythrocyte conjugates but significantly higher than the response to allogeneic conjugates.

The response of normal mice to syngeneic erythrocytes was weaker than the response of T-cell-deficient mice, which could have been caused by suppressor T cells. Their response to allogeneic conjugates was higher than the response of T-cell-deficient mice and the response to xenogeneic conjugates higher still. This was probably due to allo- or xenoreactive helper cells.

Received for publication 24 March 1976.

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