NATURE OF THE ANTIGENIC COMPLEX RECOGNIZED BY T LYMPHOCYTES

II. T-Cell Activation by Direct Modification of Macrophage Histocompatibility Antigens

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In a previous study we reported a technique for the in vitro generation of a primary proliferative response by guinea pig T cells to soluble protein antigens involving two consecutive cultures (1). By this procedure we found that T cells from $(2 \times 13)F_1$ guinea pigs primed with antigen-pulsed macrophages derived from one parental strain could be restimulated only with the antigen-pulsed macrophages derived from the parental strain used for initial sensitization, but not with macrophages derived from the other parental strain. This result suggests that the primary genetic restriction of the F_1 T-cell response may be imposed by the type of macrophage used for initial sensitization, irrespective of the I-region-associated (Ia)[1] antigen which that macrophage expressed. In this respect, these observations would support the concept that T cells do not recognize antigens per se, but can only be sensitized to antigen-modified membrane components or to complexes of antigen combined with certain membrane molecules. However, since F_1 T cells and parental macrophages share Ia antigens, the possibility that Ia homology is required for efficient T-cell-macrophage interactions cannot be ruled out. A direct test to differentiate between these two possibilities is to determine if T cells can be specifically sensitized with antigens presented by allogeneic macrophages. In our previous study, this was not possible, though, since T cells sensitized with antigen-pulsed allogeneic macrophages produced a substantial secondary mixed leukocyte reaction (MLR) that obscured any antigen-specific reaction that might have been present. Therefore, it was necessary to find some means of eliminating the MLR in order to detect specific T-cell priming to antigen presented on allogeneic cells.

Since it has previously been shown that the MLR induced by allogeneic macrophages can be inhibited by alloantisera directed against macrophage Ia antigens (2), we attempted to use anti-Ia sera to eliminate the MLR in the in vitro T-cell priming with antigen-pulsed allogeneic macrophages. However, in the course of these experiments we made the fortuitous observation that macro-

phages bearing anti-Ia antibodies stimulated T-cell proliferation. Under these conditions T cells could be specifically stimulated by anti-Ia-treated syngeneic or allogeneic macrophages. In addition, we found that antiserum against the B.1 antigen of the guinea pig major histocompatibility complex (MHC), the homologue of the mouse H-2K or H-2D antigens expressed by both strain 2 and strain 13 guinea pigs, also elicited T-cell proliferation and that the anti-B.1-induced stimulation appeared to be regulated by *I*-region gene products. These results are discussed with respect to the role of *I*-region gene products in the immunogenic complex recognized by T cells.

Materials and Methods

Animals. Inbred strain 2, strain 13, and $(2 \times 13)F_1$ guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md.

Preparation of Cells. Guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52; Humble Oil & Refining Co., Houston, Texas) and the resulting peritoneal exudate was harvested 3–4 days later. This cell population consisting of approximately 75% macrophages, 10% neutrophils, and 15% lymphocytes was used as a source of macrophages for in vitro priming. The unfractionated peritoneal exudate cells $(5-10 \times 10^6$ ml) were incubated for 1 h at 37°C in Hanks' balanced salt solution containing 25 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) and washed four times to remove the unbound mitomycin. A T-lymphocyte-enriched cell population was prepared by passing lymph node cells from animals injected in the foot pads several weeks previously with complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, Mich.) over a rayon wool adherence column (3).

Preparation of Alloantisera. A strain 13 anti-strain 2 (anti-Ia.2,4) serum and a strain 2 anti-strain 13 (anti-Ia.1,3) serum were prepared as previously described (4). In brief, the animals were cross-immunized with cell suspensions of lymph node and spleen cells emulsified in CFA. The animals were boosted 2 and 4 wk later by the intradermal or intraperitoneal injections of suspensions of lymph node and spleen cells in saline. 6–8 wk after primary immunization the animals were exsanguinated; the sera were heat inactivated at 56°C for 45 min and sterilized by Millipore filtration. Antisera to the B.1 alloantigen were raised in a similar manner by immunization of outbred B.2, Ia.1,3 animals with inbred strain 13 (B.1, Ia.1,3) cells. All of the cytotoxic activity could be removed from this antiserum by absorption with either inbred strain 2 or strain 13 cells. All antisera were specific by physicochemical criteria for Ia antigens or the B.1 antigen as determined by immunoprecipitation of a tritiated cell extract and analysis by polyacrylamide gel electrophoresis (kindly performed by Dr. B. D. Schwartz).

In Vitro Priming. T-cell-enriched lymph node cells (5×10^6) were primed by culturing with untreated macrophages (1×10^6) in 17×100 mm tissue culture tubes in a total vol of 1.5 ml of EHAA medium (5), containing L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 5-fluorocytosine (5 μg/ml), 2-mercaptoethanol $(5 \times 10^{-5}$ M), and 5% heat-inactivated normal guinea pig serum (NGPS) or alloantiserum. In some experiments, as indicated in the table legends, the macrophages $(2-5 \times 10^6)$ were incubated in 20–50 μl of antiserum for 30 min at 37°C; the antiserum-treated macrophages were then washed twice to remove the unbound antiserum and mixed with T lymphocytes. During the primary culture the cells were incubated for 1 wk at 37°C in 2% CO_2 in air. On the 3rd day of the first culture the medium was decanted and replaced with 1.5 ml of fresh EHAA medium containing 5% NGPS or alloantiserum.

In Vitro Assay of DNA Synthesis. After the 7-day initial culture approximately 20–30% of the original cell number was recovered and the cells were approximately 95% viable as determined by trypan blue exclusion. The antigen-primed T cells $(1-2 \times 10^5$ cells per well) recovered from the first culture were restimulated in the second culture in microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Arlington, Va.) with fresh peritoneal exudate cells $(1 \times 10^5$ per well) in a total vol of 0.2 ml of EHAA medium containing 5% NPGS or alloantiserum. In some experiments the stimulator cells were treated with antiserum before culture as described above. After incubation for 2 days at 37°C in 5% CO_2 in air, 1 μCi of tritiated thymidine (^{3}H-TdR, specific activity 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each well. The amount of radioactivity incorporated into cellular DNA was determined after an additional 18-h incuba-

tion with the aid of a semiautomated microharvesting device (6). The results of triplicate cultures are expressed as either total counts per minute per culture (cpm) or as the difference between antigen-stimulated and nonstimulated control cultures (Δ cpm).

Results

T-Lymphocyte Activation by Anti-Ia Antiserum.   When strain 13 T cells were incubated with syngeneic macrophages in the presence of 2 anti-13 alloantiserum in the first culture and transferred to fresh strain 13 macrophages in the presence of 2 anti-13 antiserum in the second culture substantial T-cell activation occurred, as measured by increased DNA synthesis (Table I, line 4). This stimulation appeared to be specific for 2 anti-13-induced T-cell priming since no activation occurred when 2 anti-13 sera was included in only the first (Table I, line 3) or second (Table I, line 2) cultures. Similarly, no stimulation was observed when NGPS was included in both cultures (Table I, line 1). Since the 2 anti-13 antiserum is directed only against the strain 13 Ia antigens, this stimulation results either from direct antibody modification of Ia or from priming to anti-Ia idiotypic determinants. The former was suggested by the failure of 13 anti-2 alloantiserum in the first and second cultures to activate strain 13 T cells indicating that activation results from anti-Ia binding to the T cells or macrophages and not nonspecific absorption of alloantibodies (Table I, lines 6 and 7). The failure of 2 anti-13 antiserum to activate T cells in the presence of strain 2 macrophages in the second culture (Table I, line 5) indicates that this antibody-induced stimulation results from antibody binding to the macrophage rather than by direct action on the T cell. This was substantiated by the fact that 2 anti-13-pulsed macrophages were efficient stimulators and by experiments in which T cells were specifically activated by alloantisera directed only against allogeneic macrophages (see below).

T-Lymphocyte Activation by Anti-Ia-Treated Allogeneic Macrophages.   Previous studies have shown that the response of guinea pig T lymphocytes stimulated with allogeneic macrophages in a MLR could be dramatically reduced by anti-Ia serum directed against the macrophage (2). By this technique we were able to demonstrate that T cells could be specifically activated by anti-Ia bound to allogeneic macrophages (Table II). When strain 13 T cells were stimulated with allogeneic strain 2 macrophages in the presence of NGPS in the first and second cultures a substantial secondary MLR was observed (Table II, line 2). If 13 anti-2 antiserum was included in the first culture but replaced with NGPS in the second culture, the secondary MLR was reduced approximately 80% (Table II, line 3). However, if 13 anti-2 antiserum was included in both the first and second cultures a substantial increase in T-cell proliferation was seen which approximated that seen in the uninhibited secondary MLR (Table II, line 4). Similar results were obtained with strain 2 T cells stimulated with strain 13 macrophages in the presence of 2 anti-13 antiserum (Table II, bottom half). These results suggest that at the same time that the MLR is blocked by anti-Ia sera directed against the macrophage, T cells are also specifically sensitized to the anti-Ia antibodies bound to the allogeneic macrophage.

T-Lymphocyte Activation by Anti-B.1 Antiserum.   Since antibodies directed solely against macrophage Ia antigens could activate T cells it became important to determine if antibodies directed against other membrane components

TABLE I

Stimulation of Strain 13 T Lymphocytes by 2 Anti-13 Alloantiserum

First culture*			Second culture‡		
T lympho-cyte	Macro-phages	Sera	Macro-phages	Sera	³H-TdR cpm
13	13	NGPS	13	NGPS	1,580
13	13	NGPS	13	2 Anti-13	2,370
13	13	2 Anti-13	13	NGPS	700
13	13	2 Anti-13	13	2 Anti-13	23,800
13	13	2 Anti-13	2	2 Anti-13	3,390
13	13	13 Anti-2	13	13 Anti-2	1,540
13	13	13 Anti-2	2	13 Anti-2	1,160

* Strain 13 T lymphocytes were incubated with strain 13 macrophages for 7 days in the presence of 5% 2 anti-13 or 13 anti-2 serum as indicated.

‡ The incorporation of ³H-TdR was determined 4 days after transferring the cells recovered from the first cultures to fresh strain 13 or strain 2 macrophages in the presence of 5% 2 anti-13 or 13 anti-2 serum in microtiter wells.

caused similar stimulation. We found that strain 13 T cells cultured with syngeneic macrophages in the presence of antiserum directed against the B.1 antigen of the guinea pig MHC, the homologue of the mouse H-$2K$ and/or H-$2D$ antigens, in both the first and second cultures were also activated (Table III). This activation was specific for anti-B.1 since T cells primed with anti-B.1-treated macrophages in the first culture could not be restimulated by 2 anti-13-treated macrophages in the second culture. In similar fashion, strain 13 T cells primed with 2 anti-13-treated macrophages in the first culture were incapable of being restimulated by anti-B.1-treated macrophages in the second culture but showed a good secondary response with 2 anti-13-treated macrophages. However, if the T cells were primed with macrophages simultaneously treated with both 2 anti-13 and anti-B.1 antisera in the first culture they could be restimulated with either 2 anti-13 or anti-B.1-treated macrophages in the second culture.

Genetic Restriction on the T-Lymphocyte Activation by Anti-B.1 Antiserum. Although T cells could be specifically activated by anti-B.1-treated macrophages we found that this response was restricted to the histocompatibility type of the macrophage used for initial sensitization (Table IV). If $(2 \times 13)F_1$ T cells were stimulated with anti-B.1-treated macrophages from one parent in the first culture they could only be restimulated with the anti-B.1-treated macrophage used for the initial sensitization, and not with those of the other parent despite the fact that both parents are B.1 positive. For example, $(2 \times 13)F_1$ T cells primed with anti-B.1-treated strain 13 macrophages in the first culture could only be restimulated with anti-B.1-treated strain 13 or $(2 \times 13)F_1$, but not strain 2, macrophages in the second culture. In similar fashion, $(2 \times 13)F_1$ T cells primed with anti-B.1-treated strain 2 macrophages in the first culture could only be restimulated with strain 2 or $(2 \times 13)F_1$, but not strain 13, anti-B.1-treated macrophages. F_1 T cells initially sensitized with anti-B.1-treated $(2 \times 13)F_1$ macrophages could be restimulated with anti-B.1-treated macrophages from either parent.

TABLE II

Stimulation of T Lymphocytes by Anti-Ia Sera Directed against Allogenic Macrophages

First culture*			Second culture‡		^{3}H-TdR cpm	
T lym-phocyte	Macro-phages	Sera	Macro-phages	Sera	Exp. 1	Exp. 2
13	2	NGPS	13	NGPS	4,070	268
13	2	NGPS	2	NGPS	142,700	181,180
13	2	13 Anti-2	2	NGPS	32,030	45,780
13	2	13 Anti-2	2	13 Anti-2	82,000	188,000
2	13	NGPS	2	NGPS	3,600	339
2	13	NGPS	13	NGPS	ND§	10,560
2	13	2 Anti-13	13	NGPS	30,700	1,547
2	13	2 Anti-13	13	2 Anti-13	116,300	13,310

*,‡ Same as in the footnotes for Table I.
§ ND, not determined.

Discussion

The MHC includes a number of genes which control or regulate the immune response. Previous studies have shown the *I*-region gene products play an important role for effective interaction between T cells and macrophages (7–10). Although the nature of this collaboration is at present unknown one possibility is that antigen may specifically bind to the macrophage Ia products and create an immunogenic complex recognized by T cells as proposed by Shevach (10). This concept is supported by our finding that antibody bound solely to macrophage Ia antigens is capable of sensitizing T cells. Although we could not determine the nature of this antibody-induced stimulation, two possibilities are suggested. One possibility is that the binding of antibody to the Ia antigen induces a change in the Ia molecule which T cells recognize as an altered-self immunogen. Another possibility is that T cells react against idiotypic determinants of the anti-Ia antibody which are focused on the macrophage Ia antigens.

In addition to our finding that the anti-Ia antibodies bound to syngenic macrophages was stimulatory we were also able to use this technique to specifically activate T cells with anti-Ia-treated allogeneic macrophages. This was made possible by the fact that anti-Ia antiserum directed against the allogeneic macrophage will block much of the MLR, presumably by interfering with Ia recognition by T cells (2). Our results showed that at the same time that anti-Ia antiserum directed against the allogeneic macrophage blocks the MLR, the T cells also become specifically sensitized to the anti-Ia antibodies bound to allogeneic macrophages. We interpret this finding as a demonstration that T cells can be specifically activated by antigens associated with allogeneic *I*-region gene products if they are initially sensitized with the allogeneic immunogen. These results support the concept that T-cell-macrophage interactions do not require shared MHC gene products, but that T cells recognize antigens associated with the macrophage histocompatibility type with which they were initially sensitized (1). Further evidence for this concept is our finding that strain

TABLE III

Stimulation of Strain 13 T Lymphocytes by Anti-B.1 Serum

| First culture* | Second culture,‡ ³H-TdR cpm | |
| | Sera | |
Sera	2 Anti-13	Anti-B.1
2 Anti-13	69,340	1,910
Anti-B.1	1,950	10,550
2 Anti-13 and anti-B.1	110,310	39,230

* Strain 13 T lymphocytes were incubated for 7 days with strain 13 macro-
phages pulsed with 2 anti-13 serum, anti-B.1 serum, or both, as described in
the Materials and Methods.

‡ The incorporation of ³H-TdR was determined 3 days after transferring the
cells recovered from the first culture to fresh 2 anti-13 or anti-B.1-pulsed
strain 13 macrophages in microtiter wells as described in the Materials and
Methods.

13 T cells rendered unresponsive to strain 2 alloantigens by bromodeoxyuridine
and light treatment can be specifically sensitized with hapten-modified strain 2
macrophages.[2] Several groups of investigators have reached similar conclusions
for the genetic requirements of lymphocyte-macrophage interactions in the
mouse (11-13). In all of these studies it was found that mouse T cells recognize
antigen associated only with macrophages of the same histocompatibility type
used for the initial sensitization.

Another intriguing observation is our finding that antisera directed against
the B.1 antigen of the guinea pig MHC, the homologue of the mouse H-2K and
H-2D antigens, also activated T cells in a manner similar to that found with
anti-Ia antisera. This activation was specific for the anti-B.1-sera since there
was no cross-reaction with the anti-Ia-induced activation and indicates that the
anti-Ia and anti-B.1 sera do not cause a generalized nonspecific modification of
the stimulator cells.

Another related and surprising finding was that the T-cell activation to the
anti-B.1-sera was genetically restricted to the histocompatibility type of the
anti-B.1-treated macrophage used for the initial sensitization. Since the B.1
antigens expressed by the strain 2 and strain 13 guinea pigs are serologically
identical it was anticipated that $(2 \times 13)F_1$ T cells initially sensitized with anti-
B.1-treated macrophages from one parent might have been restimulated with
anti-B.1-treated macrophages from either parent. Instead, we found that $(2 \times 13)F_1$ T cells sensitized with anti-B.1-treated parental macrophages could be
restimulated only with anti-B.1-treated macrophages of the parental type used
for the initial sensitization, but not with those of the other parent. Due to the
fact that the strain 2 and strain 13 MHC's differ only with respect to Ia antigens,
these results suggest that the immunogenic complex recognized by the T cells
may consist of the antisera-bound B.1 in association with Ia antigens. It should
be noted that although the strain 2 and strain 13 antigens are serologically

² Thomas, D. W., and E. M. Shevach. Nature of the antigenic complex recognized by T
lymphocytes. III. Specific T cell sensitization by antigens associated with allogeneic macrophages.
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TABLE IV

Genetic Restriction on the Response of (2 × 13)F₁ T Lymphocytes to Anti-B.1-Treated Parental Macrophages

First culture*		Second culture,‡ ³H-TdR Δ cpm		
		Anti-B.1-pulsed macrophages		
T lymphocyte	Anti-B.1-pulsed macrophages	(2 × 13)F₁	2	13
Exp. 1, (2 × 13)F₁	(2 × 13)F₁	17,530	3,950	42,710
	2	10,430	6,610	2,970
	13	20,790	0	38,800
Exp. 2, (2 × 13)F₁	(2 × 13)F₁	3,280	2,720	5,500
	13	5,650	830	8,620
Exp. 3, (2 × 13)F₁	2	ND§	19,680	1,360
	13		0	48,610

* (2 × 13)F₁ T lymphocytes were incubated 7 days with anti-B.1-pulsed (2 × 13)F₁, strain 2, or strain 13 macrophages as described in the Materials and Methods.

‡ The incorporation of ³H-TdR was determined 3 days after transferring the cells recovered from the first culture to fresh anti-B.1-pulsed (2 × 13)F₁, strain 2, or strain 13 macrophages in microtiter wells as described in the Materials and Methods.

§ ND, not determined.

identical at the present time, it is possible that the B.1 antigens expressed by the strain 2 guinea pigs are structurally different from the B.1 antigens expressed by strain 13 guinea pigs. In addition, while the strain 2 and strain 13 B.1 antigens are serologically identical, T cells may recognize differences which are not detected by antibody. Another possible explanation for these results may be that while strain 2 and strain 13 guinea pigs differ primarily by Ia antigens of the MHC, there are also other genetic differences between these strains. Thus, the genetic restriction of the F₁ T-cell response to anti-B.1-treated parental macrophages may actually reflect differences at genetic loci other than the *I* region. To test this possibility experiments are in progress using anti-B.1-treated macrophages from outbred animals with different Ia antigens than the immunizing strain to determine if the response is regulated by Ia antigens or by other genetic loci unrelated to the *I*-region.

There is ample evidence that the products of the MHC are capable of functional interaction with other cell surface molecules. Bevan (14) has shown that mouse cytotoxic T cells directed against minor alloantigens must share the same allele at either the *H-2K* or *H-2D* locus with the target cell. Similarly, several other investigators (15–17) have demonstrated an association between MHC antigens and tumor-specific transplantation antigens. Although the chemical basis for these molecular interactions is as yet unknown, it is not too surprising that a similar process may be mediated by *I*-region antigens on other MHC antigens. One might speculate that a direct physical linkage occurs between the Ia and modified B.1 antigens or possibly that the antisera-modified B.1 antigen renders it susceptible to further modification by the *I*-region gene product, perhaps through some enzymatic mechanism. Two possibilities for the function of *I*-region gene products suggested by our studies are that Ia antigens may

serve to directly present antigens associated with the Ia molecule, and to indirectly aid in the presentation of antigens associated with other membrane components, such as the B.1 antigens.

Our observation that antibodies bound to stimulator cells to produce an immunogenic complex that sensitizes T lymphocytes provides a novel approach for studying the nature of the antigenic complex recognized by T cells. It is thus possible to selectively modify membrane antigens and determine if the specific antibody is capable of sensitizing T cells and which type of membrane antigens are included in the antisera-induced immunogenic complex. In this manner we have shown that antibodies bound to both Ia or B.1 antigens of the guinea pig MHC on the stimulator macrophage specifically sensitizes T cells. One clinical implication of these findings may be that antibodies produced against self components including MHC antigens in some autoimmune disease states may elicit a response against the antibody-self complex and thereby perpetuate the autoimmune reaction (18).

Summary

In order to analyze the molecular structures involved in T-cell recognition we developed an in vitro primary response against alloantisera bound to histocompatibility antigens in which nonimmune guinea pig T cells can be sensitized and subsequently challenged in tissue culture with antisera-treated macrophages. If macrophages were incubated with alloantisera directed against the I-region-associated (Ia) antigens of the guinea pig major histocompatibility complex (MHC) T cells could be sensitized to the antisera bound to macrophage Ia determinants. Anti-Ia-treated syngeneic macrophages in the first and second cultures elicited specific T-cell activation, as measured by increased DNA synthesis, to the antisera-induced immunogenic determinants. Similarly, anti-Ia-treated allogeneic macrophages also specifically stimulated T cells to antisera bound to allogeneic Ia determinants while reducing the mixed leukocyte reaction. Antisera to the B.1 antigens of the guinea pig MHC, the homologue of the mouse H-2K or H-2D antigens, also elicited specific T-cell activation that did not cross-react with that produced by the anti-Ia alloantisera. Furthermore, the anti-B.1-induced stimulation appeared to be associated with the Ia antigens of the macrophage used for priming since $(2 \times 13)F_1$ T cells sensitized with anti-B.1-treated parental macrophages could be restimulated only with the parental macrophage used for initial sensitization, and not with those of the other parent. Since the parental strain 2 and strain 13 guinea pigs express serologically identical B.1 antigens and differ only by Ia antigens of the MHC, this observation suggests that both B.1 and Ia antigens may be included in the immunogenic complex recognized by T cells. However, we cannot rule out the possibility that this restriction is due to other genetic differences between strain 2 and strain 13 guinea pigs that is unrelated to the I-region. We interpret these findings as showing that macrophage Ia antigens may serve to directly present antigens bound to the Ia molecule, and possibly indirectly aid in the presentation of antigens bound to other membrane components, such as the B.1 antigens.

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