0022-1767/88/14011-3707$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 140, 3707–3711, No. 11, June 1, 1988
Printed in U.S.A.

T CELL RECOGNITION OF NONPOLYMORPHIC DETERMINANTS ON H-2 CLASS I MOLECULES[1]

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Recognition of polymorphic determinants on class I or class II MHC Ag is required for T lymphocyte responses. Using cell-size artificial membranes (pseudocytes) bearing H-2 class I Ag it is demonstrated that T cells can, in addition, recognize nonpolymorphic determinants on class I proteins. Pseudocytes bearing class I alloantigen stimulate in vitro generation of secondary allogeneic CTL responses. At a suboptimal alloantigen surface density, incorporation of class I molecules identical to those of the responder cells (self-H-2) or from third-party cells resulted in dramatically enhanced responses, whereas incorporation of class II proteins had no effect. The receptor that mediates recognition of conserved class I determinants has not been identified, but results of antibody blocking studies are consistent with the Lyt-2/3 complex of CTL having this role. Thus, class I proteins on Ag-bearing cells can have two distinct roles in T cell activation, one involving recognition of polymorphic determinants by the Ag-specific receptor and the other involving recognition of conserved determinants.

Recognition of class I and II glycoproteins of the MHC is required for activation of T cells and thus plays a central role in controlling virtually all immune system responses. Ag-dependent triggering of T cells involves interaction of the TCR with polymorphic determinants of the class I or II protein, either alone, as may be the case for alloantigen recognition, or together with foreign Ag.

Although highly polymorphic, the class I and class II Ag also possess large regions of structural invariance, and it has been suggested that recognition of nonpolymorphic determinants may also contribute to T cell triggering. Based on the good correlation between Lyt-2/3 expression on class I-restricted T cells and L3T4 expression on class II-restricted T cells, it was proposed that these molecules bind to class-specific, nonpolymorphic regions of the MHC proteins (1, 2). The results of numerous experiments examining the ability of anti-Lyt-2 antibodies to block T cell recognition and response (3–6) and the effects of transfecting cells with Lyt-2 (7, 8) are consistent with the hypothesis that it interacts with conserved class I determinants. Nevertheless, direct evidence for T cell recognition of conserved determinants by Lyt-2, or as yet unidentified surface receptors, has been lacking.

We have recently described the preparation of cell-size supported artificial membranes (pseudocytes) bearing class I Ag and their application to the study of MHC protein recognition by alloantigen-specific precursor CTL (9, 10). Stimulation of a secondary in vitro CTL response was found to be critically dependent on the surface density of H-2 alloantigen on the membrane (10). Taking advantage of this critical density dependence, we have now obtained evidence that directly demonstrates that T cells can recognize conserved class I determinants and that this recognition enhances responses resulting from interaction with specific Ag.

MATERIALS AND METHODS

Mice and tumor cells. (BALB/c × DBA/2)F_1 ($CD2F_1$), C57BL/6, and AKR/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME. RDM-4, an AKR lymphoma, P815, a mastocytoma of DBA/2 origin, and EL-4, a C57BL/6 thymoma, were maintained in tissue culture and by passage as ascites.

Purification of class I and class II Ag. MHC Ag were purified by mAb affinity chromatography as previously described in detail (11, 12). H-$2K^k$ was purified from RDM-4 tumor cell lysates using an 11-4.1 mAb column, and H-2^d and H-2^b class I Ag were purified from P815 and EL-4 cells using an M1/42 mAb column. M1/42 mAb is a rat anti-mouse class I-specific antibody (12). I-A^k and I-E^k were purified from LPS blasts using 10-2.16 and 14-4-4S mAb columns, respectively (13). All Ag preparations were analyzed by SDS-PAGE using Coomassie blue staining to visualize the protein, and protein concentration was determined using BSA as the standard, as previously described (11–13).

Preparation of pseudocytes. Cell-size Ag-bearing artificial membranes were prepared as previously described in detail (9). Briefly, Spherisorb 5-μm ODS1 beads (Phase Sep, Norwalk, CT) were suspended by vortex mixing in 0.5% deoxycholate in 10 mM Tris buffer, 0.14M NaCl, pH 8.0, washed twice in this buffer by centrifugation (2 min at 1500 rpm), and counted using a hemocytometer. Lipids were obtained by chloroform/methanol extraction of P815 cells and quantitated based on phosphate (14). Components were incorporated onto beads by mixing protein, lipid, and beads in deoxycholate/Tris/NaCl and dialyzing at 4°C for 36 to 48 h to remove detergent. After dialysis, the beads were washed three times in sterile medium containing FCS and stored at 4°C until added to culture. Incorporation onto beads was assessed using ^{125}I-labeled protein, and surface exposure of protein was determined by papain cleavage as previously described (9, 10). The beads were counted with a hemocytometer.

Ag density was varied by varying the ratio of protein to beads in the starting mixture. Protein surface density is expressed as relative densities, with a relative density of 1× corresponding to 1.2 μg of protein per 10^7 beads. Over the range examined (up to 10×, i.e., 12 μg/10^7 beads), about 40% of input protein was exposed at the bead surface as assessed by papain cleavage.

In vitro CTL generation. Responder splenocytes from $CD2F_1$ or C57BL/6 mice, primed 2 to 6 mo previously by i.p. injection of 2×10^7 RDM-4 (H-2^k) cells, were placed into culture with irradiated allogeneic stimulator cells or pseudocytes in 2 ml of medium with 7×10^6 responder cells/well (Linbro, New Haven, CT) at 37°C and 5% CO_2. An optimal concentration of Con A supernatant, an $(NH_4)_2SO_4$

Received for publication December 8, 1987.
Accepted for publication March 9, 1988.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by American Cancer Society, Inc. Grant IM-415 and National Institutes of Health Grant RO1 CA43062. S.A.N.G. was supported in part by National Heart, Lung and Blood Institute Grant HL-07374 while an M.D.-Ph.D. candidate in the Division of Medical Sciences, Committee on Immunology, Harvard Medical School, Boston, MA.

fraction of supernatant fluid from rat spleen cells cultured with Con A (14), was added 22 h after initiation of the cultures. Details of culture medium and preparation of allogeneic spleen stimulator cells have been described (10).

After 5 days, cultures were assayed for cytolytic activity at several E/T ratios by triplicate determination of the ^{51}Cr released in 4 h from 10^4 labeled RDM-4 or P815 target cells. Percent specific release was calculated as 100 × (experimental − spontaneous ^{51}Cr release)/(total released by 0.5% deoxycholate − spontaneous ^{51}Cr release). Spontaneous release was less than 10% in all experiments. One lytic unit is defined as the number of effectors required to lyse 50% of the target cells in 4 h, and results are expressed as lytic units per 10^6 cells.

Anti-Lyt-2 mAb inhibition of effector CTL generation was studied using the 53-6.7 mAb (15) and, as a control, the HO-13-4 anti-Thy-1.2 mAb (16), as described previously (10). Briefly, responder splenocytes were treated with mAb for 60 min at room temperature. Alloantigen-bearing stimulator cells or pseudocytes were then added and culturing was begun. Con A supernatant addition at 24 h and assay of lytic activity after 5 days were performed as described above. Dose-response titrations showed 0.45 μg/culture well of 53-6.7 to maximally inhibit responses to spleen stimulator cells and this amount of 53-6.7 and HO-13-4 were used in all experiments. This level of 53-6.7 mAb was found not to block lysis when added directly to the chromium release assay (10). Thus, inhibition of responses by antibody added at the initiation of culture did not result from blocking of effector cell activity by antibody, which might remain after 5 days of culture and washes before the lytic assay. All experiments included controls using HO-13-4, which in all cases resulted in less than 10% decrease in the response level.

RESULTS

Previous work showed that alloantigen-bearing pseudocytes stimulated generation of specific secondary in vitro CTL responses. Response is completely dependent on recognition of the alloantigen on the bead, and the resultant effectors lyse only target cells that bear the relevant Ag (9). Response to alloantigen on pseudocytes is also completely dependent upon addition of lymphokines (Con A supernatant) to the cultures (9). In all of the experiments described here, an optimum concentration of Con A supernatant was added to the cultures, and controls lacking Ag were done to insure that all responses measured were dependent on Ag recognition.

Generation of a response was found to be critically dependent on the surface density of alloantigen on the pseudocytes (10). Beads prepared by incorporation of 1.2 μg of H-2K^k per 10^7 beads (arbitrarily defined as a relative H-2 Ag density of 1×), stimulated very little response, whereas increasing the Ag density just fourfold stimulated a maximal response. When the responses to beads of differing Ag densities were examined by varying the dose of particles added to cultures, maximal responses were observed in all cases with 10^6 beads/well (10), the number used in the experiments reported here. Thus, the maximal response reached with high density beads (5×) was never achieved by addition of low (2×) density beads at any dose. Whereas the magnitude of maximal response varied between groups of animals (experiments), submaximal responses were always seen using alloantigen at a relative density of 2× or less, and a relative density of 4× or greater gave maximal responses. Figure 1 shows this comparison for 3 experiments, representative of a total of 10 experiments performed. Based on FACS analysis (10) and ELISA assay (K. Kane and M. Mescher, unpublished observations), the range of class I surface protein densities on normal cells is similar to that of pseudocytes in the 2× to 5× relative density range.

The specificity and marked density dependence of the response to pseudocytes made it possible to determine

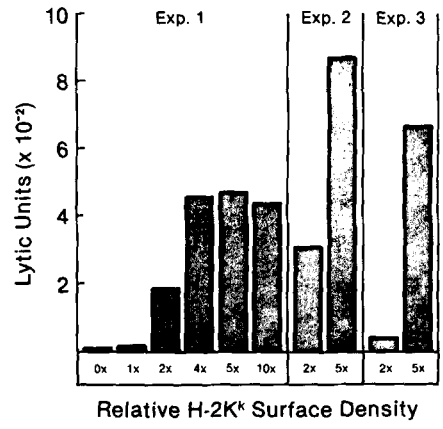

Figure 1. The effect of increasing class I alloantigen surface density on generation of a CTL response. Pseudocytes were prepared using 0, 1.2 (1×), 2.4 (2×), 3.6 (3×), 4.8 (4×), 6 (5×), or 12 μg (10×) of H-2K^k per 10^7 beads; numbers in parenthesis refer to relative surface densities on an arbitrary scale. Splenocytes from CD2F$_1$ (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 (H-2^k) cells were cultured with 10^6 pseudocytes/culture for 5 days as described in *Materials and Methods*. Lytic activity was measured at the end of 5 days using ^{51}Cr-labeled RDM-4 target cells and the results are expressed as lytic units/10^6 cells.

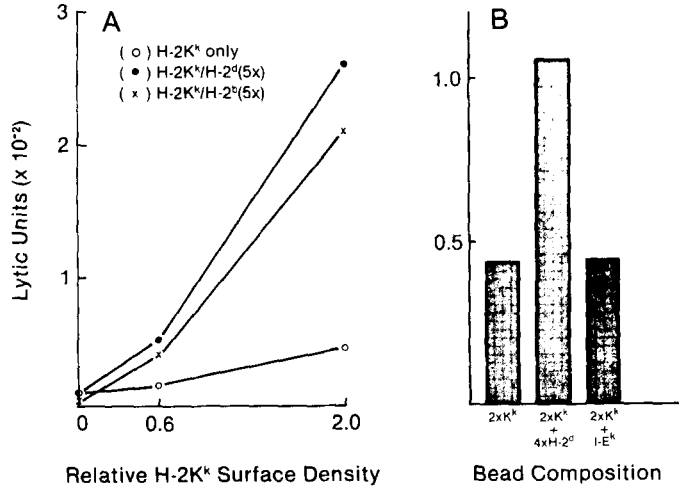

Figure 2. Incorporation of self or third party class I proteins augments the CTL response to suboptimal densities of alloantigen. *A*, Pseudocytes were prepared using 0 (0×), 0.72 (0.6×), or 2.4 μg of (2×) H-2K^k per 10^7 beads with either no other protein (O), 6 μg of H-2^b (×), or 6 μg of H-2^d (●) per 10^7 beads. Responder splenocytes from C57BL/6 (H-2^d) mice previously immunized with RDM-4 (H-2^k) were cultured for 5 days with 10^6 pseudocytes/culture and lytic activity was determined using RDM-4 target cells. *B*, Pseudocytes were prepared using 2.4 μg (2×) of H-2K^k per 10^7 beads, with either no additional protein, 6 μg of H-2^d, or 6 μg of I-E^k per 10^7 beads. Responder splenocytes from CD2F$_1$ (H-2^d) mice previously immunized with RDM-4 (H-2^k) were cultured for 5 days with 10^6 pseudocytes/culture and lytic activity was determined using RDM-4 target cells.

whether class I molecules lacking the appropriate polymorphic (allogeneic) determinants could nevertheless contribute to an effective interaction. Pseudocytes were prepared that had either low density alloantigen or low density alloantigen along with either self or third-party class I protein. Suboptimal responses to low density alloantigen were dramatically augmented if self or third-party class I protein was present on the same membrane (Fig. 2*A*), whereas beads bearing no alloantigen stimulated no response regardless of the presence of self or third-party class I protein.

Enhancement of suboptimal responses was specific for class I MHC proteins. In all experiments, the presence of 5× levels of self or third-party class I protein on beads bearing suboptimal alloantigen augmented responses

(Fig. 2*A*; Table I). In contrast, no enhancement was observed when the same amount of class II I-E^k (Fig. 2*B*) or I-A^k (data not shown) were incorporated (with the exception of one experiment in which incorporation of class II resulted in a 25% increase over the level of response obtained with alloantigen alone). The class I-specific enhancement occurred only if alloantigen and the self or third party proteins were on the same bead surface. Adding beads bearing just self or third-party H-2 proteins to cultures containing low density alloantigen beads resulted in no greater response than that obtained when only the alloantigen-bearing beads were present (Table I, *bottom line*).

Enhancement of the response to suboptimal alloantigen was dependent on the surface density of the self or third-party class I protein. Augmentation was negligible at a relative density of 0.6× (data not shown) but apparent at 2× and maximal at 5× relative densities (Table I). The same results were found when either H-2^b anti-H-2^k or H-2^d anti-H-2^k responses were examined. Although class I of the responder haplotype showed slightly greater augmentation than third-party class I in the experiment shown in Figure 2*A*, no reproducible differences in augmentation by self vs third-party class I proteins have been found.

The above results provided evidence that precursor CTL bear a receptor, distinct from the Ag-specific TCR, that interacts with conserved determinants of class I proteins on the Ag-bearing membrane. The Lyt-2/3 complex is the likely candidate as the receptor that mediates this recognition. We therefore examined the ability of anti-Lyt-2 antibody to block responses to pseudocytes having varying surface compositions. Anti-Lyt-2 was added to cultures at their initiation, using an amount of antibody previously shown to maximally block generation of responses but to have no blocking effect on target cell lysis when added to a ^{51}Cr release assay (10). Anti-Lyt-2 completely blocked responses to beads bearing a low (2×) density of alloantigen, whereas responses to beads bearing a high (5×) alloantigen density could be only partially blocked (Table I). However, whereas responses to beads bearing low density alloantigen (2×) plus self class I (5×) were comparable in magnitude to those obtained using high density alloantigen (5×), they were significantly more susceptible to blocking by anti-Lyt-2 antibody (Table I). Thus, a maximal response obtained by self or third-party class I augmentation was more susceptible to blockade by anti-Lyt-2 antibody than was the same maximal response when obtained by increasing alloantigen density.

DISCUSSION

Triggering of precursor CTL by artificial membranes bearing a suboptimal alloantigen surface density is markedly augmented if self or third-party class I, but not class II, proteins are present on the same membrane (Fig. 2; Table I). These results provide strong evidence that precursor CTL bear a receptor that interacts with nonpolymorphic determinants of class I proteins on the Ag-bearing membrane. The augmenting effect of class I protein occurs equally with self or third-party H-2 molecules and thus is not self-restricted. The lack of response in the absence of alloantigen also indicates that cross-reactive recognition is not involved (Table I; Fig. 2). Thus, it appears very likely that the receptor for conserved determinants is distinct from the Ag-specific TCR. Class I binding by the conserved determinant receptor might augment responses by simply strengthening the avidity of the cell-cell (cell-pseudocyte) interaction, thus promoting interactions between Ag and TCR. Alternatively, the conserved determinant receptor might, itself, be involved in transmembrane signal generation.

Although this report presents the first direct evidence for recognition of conserved class I determinants, such recognition has been previously postulated to occur (1). Antibodies specific for the Lyt-2/3 protein complex present on the surface of murine CTL can block CTL responses to Ag. The extent of blocking obtained is variable and depends on both the CTL clone or population examined and on the target cell used (3–6). Antibody blocking effects, and the correlation of expression of Lyt-2/3 on class I-restricted T cells and L3T4 on class II-restricted T cells, led to the suggestion that these surface proteins might bind conserved determinants on class I and class II proteins, respectively (1, 2). Antibody-blocking effects can be difficult to interpret, but strong additional support for a role for Lyt-2 in augmenting Ag-specific cell interactions in an MHC class-specific manner has recently been provided by experiments examining the effects of transfecting the gene for this protein into T cells lacking the protein (7, 8).

Examination of the effects of anti-Lyt-2 blocking of responses to class I proteins on pseudocytes yielded results consistent with the suggestion that Lyt-2 mediates

TABLE I

Inhibition by anti-Lyt-2 antibody of precursor CTL recognition of class I MHC proteins on pseudocytes[1]

Bead Stimulus[2]	Exp 1			Exp 2			Exp 3		
	No mAb	53-6.7	% Inhibition	No mAb	53-6.7	% Inhibition	No mAb	53-6.7	% Inhibition
H-2K^k(2×)	308	32	90	217	17	92	330	17	95
H-2K^k(5×)	870	526	39	488	227	53	—[3]	—	
H-2^d (5×)	—	—		—	—		10	—	
H-2K^k/H-2^d(2×)/(2×)	—	—		357	34	90	—	—	
H-2K^k/H-2^d(2×)/(5×)	1,053	392	63	488	125	74	500	45	91
H-2K^k(2×) + H-2^d(5×)[4]	—	—		—	—		317	13	96

[1] Splenocytes from CD2F$_1$ (H-2^d) mice previously primed with RDM-4 (H-2^k) were cultured for 5 days in the presence of pseudocytes of the indicated composition and lytic activity assayed using RDM-4 targets. When used, mAb was preincubated with responder splenocytes for 60 min before addition of pseudocytes. Controls using anti-Thy 1.2 (HO-13-4) were performed in all experiments and always resulted in less than 10% inhibition. Cytolytic activity is expressed as lytic units per 10^6 cells.

[2] Pseudocytes having the indicated surface composition were prepared as described in *Materials and Methods* and used at 10^6 beads/culture.

[3] Not determined.

[4] In this case two separate pseudocyte preparations were made, one having only H-2K^k and one having only H-2^d. Then, 10^6 of each type were added to the same culture wells.

the demonstrated class I conserved determinant recognition (Table I). Some experimental evidence has been presented to suggest that blocking effects of anti-Lyt-2 and anti-L3T4 might result from antibody cross-linking of these proteins delivering a negative signal to the T cell rather than from blocking of interaction with a ligand on the Ag-bearing cell (17–20). Although this possibility cannot be ruled out in the experiments shown here, it would appear to be an unlikely explanation of the results. The level of response to low density alloantigen plus self or third-party class I is equal to or greater than that obtained with higher density alloantigen, yet anti-Lyt-2 is significantly more inhibitory in the former case (Table I). Thus, a "negative" signal mediated by anti-Lyt-2 would have to have differing effects, despite comparable levels of "positive" signals in the two cases. The alternative explanation, that Lyt-2/3 mediates the demonstrated augmentation by class I conserved determinant recognition and that anti-Lyt-2 blocks this interaction, would appear more likely.

The postulated role of Lyt-2/3 interaction with conserved class I determinants has recently been extended by the further suggestion that it may bind to the same class I molecule that is bound by the Ag-specific receptor to form a multimolecular complex needed for effective transmembrane triggering (21–23). Based on antibody blocking and triggering effects (23, 24) a similar role for L3T4 has been proposed, and some evidence for Ag-induced association of L3T4 with the TCR complex has been obtained by examining their coclustering (25) and comodulation (24) on T cells. The results described here do not rule out this possibility. They do argue, however, that it is not the only mechanism for enhanced response via conserved determinant recognition. Self or third-party class I molecules can serve to augment, whereas only the class I alloantigen is bound by the Ag-specific TCR with sufficient affinity to result in signal generation leading to a response.

Interaction of CTL with target cells occurs over relatively large areas of the cell surfaces (26). The strong dependence of precursor CTL recognition and triggering on class I Ag multivalency (27) and surface Ag density (10) is consistent with a model in which numerous low affinity interactions combine to result in sufficiently high avidity for binding and triggering to occur. The results described in this report demonstrate that these interactions with class I proteins are of at least two types, recognition of polymorphic determinants by the Ag-specific TCR and recognition of conserved determinants. Regardless of whether conserved determinant recognition is mediated by Lyt-2/3 proteins, as is suggested by the antibody blocking results, or by an as yet unidentified receptor it is clear that this recognition can be a critical factor in determining whether a CTL response to suboptimal Ag will occur. Using the augmentation assay described here and class I Ag that have been biochemically or genetically modified, it should be possible to identify the invariant structural regions involved in this recognition.

Acknowledgments. We thank Paul Champoux for excellent technical assistance. This is publication 113 from the Medical Biology Institute.

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