Proc. Natl. Acad. Sci. USA
Vol. 74, No. 3, pp. 1229–1233, March 1977
Immunology

Cytolytic thymus-derived lymphocytes specific for allogeneic stimulator cells crossreact with chemically modified syngeneic cells

(*H-2* loci/major histocompatibility complex/trinitrophenyl-syngeneic targets/transplantation antigens)

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ABSTRACT Mouse spleen cells cocultured with irradiated allogeneic stimulator cells develop cytolytic effector cells capable of lysing ^{51}Cr-labeled syngeneic trinitrophenyl-derivatized tumor or spleen targets and to a lesser degree unconjugated tumor cells in addition to the allogeneic stimulator cells. Lysis of trinitrophenyl-syngeneic targets was inhibited competitively by cold trinitrophenyl-syngeneic tumor or spleen targets as well as by cells bearing the allogeneic stimulator H-2 haplotype demonstrating the immunological specificity of the interaction. Allogeneic H-2 specificities may, therefore, be considered variants of modified autologous H-2 specificities against which cytolytic thymus-derived clones potentially exist that are capable of exerting immunological surveillance.

The specificity of cytolytic thymus-derived lymphocytes (CTL) is directed to products of the major histocompatibility complex (MHC) (1). In the mouse, the most extensively investigated experimental species in this respect, the products of the *H-2K* and/or *H-2D* loci are the preferred targets of CTL (2), although, when appropriate strains differing only at the *I* region of the MHC are used, CTL against *I* region controlled specificities can be demonstrated (3, 4). This *H-2* restriction of CTL specificity has been observed initially for allogeneic immunizations and later extended to xenogeneic immunizations (ref. 5 and S. J. Burakoff, M. E. Dorf, and B. Benacerraf, submitted for publication).

However, CTL responses can also be raised to syngeneic cells, virally infected or chemically modified. The specificity of these CTL, although encompassing in each case the particular viral or chemical modifier, is nevertheless again directed against products of the *H-2K* and/or *H-2D* loci (6, 7). Thus, CTL effector cells generated to trinitrophenyl (N_3ph)-conjugated syngeneic spleen cells lyse preferentially N_3ph-syngeneic targets (7) and crossreactively lyse N_3ph-allogeneic targets, to a lesser extent (8, 9). The hypothesis has been proposed that CTL clones raised against virally infected or chemically modified syngeneic targets have receptors specific for slight modifications of the products of the autologous *H-2K* or *H-2D* loci (6, 7). Possibly, but not necessarily, there may be additional receptors specific for the modifier viral or chemical agents (6).

The MHC molecules initially identified as responsible for transplantation immunity should more realistically be considered to have evolved as the appropriate targets for T (thymus-derived) lymphocytes responsible for immunological surveillance and capable of detecting cells which exhibit the slightest modification from self on their membrane as a result of (*i*) the expression of viral antigens, (*ii*) chemical modification, and (*iii*) malignant transformation. In favor of this interpretation is the demonstration that the interaction of CTL specific for tumor specific transplantation antigens (TSTA) on syngeneic target cells is inhibited by alloantisera directed to *H-2K* and/or *H-2D* specificities (10, 11). To maximize the effectiveness of this critical defense mechanism in higher organisms, a relatively large population of T lymphocytes are, therefore, committed to react with a restricted family of MHC molecules evolved for this purpose. Considering the specificity of CTL responses in this light one would postulate that: (*i*) the alloreactive cells responsible for tissue rejection are the inevitable but unwanted consequence of the CTL surveillance mechanism against modified self, (*ii*) extensive crossreaction should be observed among CTL specific for modified self and for allogeneic targets, (*iii*) CTL raised against allogeneic targets, should exhibit crossreactive lysis for modified syngeneic targets. Experiments will be reported which demonstrate that CTL raised against nonderivatized allogeneic stimulators crossreactively lyse N_3ph-modified syngeneic spleen or tumor targets and more weakly lyse unconjugated syngeneic malignant targets bearing TSTAs.

MATERIALS AND METHODS

Mice. Six- to twelve-week-old male or female mice of the following strains were employed for these studies: C57BL/6 [B6] (*H-2^b*), B10.Br (*H-2^k*), B10.D2 (*H-2^d*), BALB/c (*H-2^d*), and DBA/2 (*H-2^d*). All were purchased from Jackson Laboratory, Bar Harbor, Me.

Tumors. P815 (*H-2^d*) mastocytoma was maintained in DBA/2 mice, RDM-4 (*H-2^k*) was maintained in AKR mice and the EL4 (*H-2^b*) leukemia was maintained in B6 mice. These tumors were carried in ascites form by biweekly intraperitoneal transfer.

Generation of Effector Cells. Seven $\times 10^6$ spleen cells were cultured at 37° with 3×10^6 irradiated (1200R, General Electric Maximar 250-III) allogeneic spleen cells in 2 ml for 5 days in 16 mm tissue culture wells (Linbro Chemicals, New Haven, Conn.) with a humid atmosphere of 95% air and 5% CO_2 (8). Erythrocytes were removed from stimulator cells with Tris·HCl/ammonium chloride.

Cell culture medium consisted of RPMI 1640, penicillin (100 units/ml), streptomycin (100 μg/ml) (Associated Biomedic Systems) supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (Microbiological Assoc., Bethesda, Md.).

^{51}Cr Release Assay. The ^{51}Cr release assay used in this study has been described in detail (8). The assay was performed in SMEM-10 which consisted of minimum essential medium with Earle's salts with glutamine (2 mM), 1$\times$ nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml) and

Abbreviations: CTL, cytolytic thymus-derived lymphocytes; MHC, major histocompatibility complex; N_3ph, trinitrophenyl; T cell, thymus-derived lymphocyte; TSTA, tumor specific transplantation antigens; B6, C57BL/6.

Table 1. Cytolysis of syngeneic N_3ph-derivatized tumor targets by *H-2^d*-stimulated CTL*

Exp.	Responder	Stimulator	Targets†		
			EL-4-N_3ph (*H-2^b*)	EL-4	P815 (*H-2^d*)
I	C57BL/6 (*H-2^b*)	DBA/2 (*H-2^d*)	32	3	80
II	C57BL/6 (*H-2^b*)	DBA/2 (*H-2^d*)	56	15	—
III	C57BL/6 (*H-2^b*)	B10.D2 (*H-2^d*)	46	15	—
			RDM-4-N_3ph (*H-2^k*)	RDM-4	P815 (*H-2^d*)
I	B10.BR (*H-2^k*)	DBA/2 (*H-2^d*)	16	3	61
II	B10.BR (*H-2^k*)	DBA/2 (*H-2^d*)	36	15	—
III	B10.BR (*H-2^k*)	B10.D2 (*H-2^d*)	44	14	—

* Values are percent specific release of ^{51}Cr.
† Effector to target ratio was 40:1. Spontaneous release was 10–30% for EL4, 20% for RDM-4, and 20% for P815.

10% heat-inactivated fetal calf serum. Tumor or spleen targets were labeled by incubation with Na $^{51}CrO_4$ (Amersham, Arlington Heights, Ill.). Spleen cells were then centrifuged on a Ficoll-Hypaque gradient to remove dead cells and erythrocytes. Derivatization was accomplished by incubating 0.1 ml of 10 mM trinitrobenzenesulfonic acid in 0.05 M phosphate buffer (pH 7.4) with 5×10^6 cells in 0.1 ml of SMEM-10 medium for 10 min at 37°, in an atmosphere of 95% air and 5% CO_2. Then the cells were washed three times with minimum essential medium with Earle's salts to which was added 5% heat-inactivated fetal calf serum.

Fifty microliters of cell suspension containing 1×10^4 labeled tumor targets or 2×10^4 labeled spleen targets in SMEM-10 was added to 10 × 75 mm round-bottom glass tubes. In experiments involving competitive inhibition by unlabeled target cells, varying numbers of unlabeled cells in 50 µl of SMEM-10 were added just before the addition of effector cells or normal spleen cells in 100 µl. These cultures were mixed on a vortex and incubated for 4 hr at 37° in 83% N_2, 10% CO_2, and 7% O_2 on a rocking platform. After incubation, 1 ml of ice-cold minimal essential medium was added to each tube, the tubes were centrifuged at 800 × *g* for 15 min, and the supernatants removed and assayed to determine isotope release. All measurements were performed in duplicate. The standard error of the means was always <2–3%. Differences of >5% were almost invariably significant at $P < 0.05$. Percent-specific release was calculated as $(E - C)/FT - C) \times 100\%$ where E is isotope release from tubes containing immune effectors plus targets, C is isotope release from tubes containing normal spleen cells and target cells ("spontaneous release"), and FT is maximum isotope release from target cells after freeze-thawing four times.

RESULTS

Allogeneically sensitized CTL lyse N3ph-derivatized targets with *H-2* loci identical to the effectors

When normal spleen cells are cultured with irradiated allogeneic *H-2^d* spleen cells, cytotoxic effector cells are generated that are able to lyse N3ph-derivatized tumor or spleen targets that are identical to the effectors with respect to the *H-2* loci. As shown in Table 1, B6 (*H-2^b*) anti-DBA/2 (*H-2^d*) effector cells lyse N3ph derivatized EL4 (EL4-N3ph) targets to a far greater extent than underivatized EL4 targets. B10.BR (*H-2^k*) anti-DBA/2 effectors are able to lyse RDM-4-N3ph (*H-2^k*) targets more effectively than RDM-4 target cells. In addition, B6 and B10.BR CTL raised against allogeneic *H-2^d* stimulators show low but significant lysis in four out of six experiments against the syngeneic underivatized tumors EL-4 and RDM-4, respectively.

The ability of CTL raised against allogeneic targets to kill N3ph-modified syngeneic targets is not limited to tumor models. Table 2 demonstrates that B6 anti-DBA/2 CTL are able to lyse B10-N3ph (*H-2^b*) spleen targets with identical *H-2* loci and that B10.BR-N3ph spleen targets are lysed by syngeneic B10.BR anti-DBA/2 effector cells. Though some lysis of the nonderivatized *H-2*-identical tumor targets was observed, lysis of the nonderivatized *H-2*-identical spleen targets was never observed.

To estimate the extent of the crossreactivity the ability of B6 anti-DBA/2 CTL to lyse P815 (*H-2^d*) (a tumor target with *H-2* loci identical to the stimulator) was compared with their ability to lyse EL4-N3ph (Fig. 1). Semilogarithmic plots were constructed of "percent specific ^{51}Cr-release" vs. effector/target

Table 2. Cytolysis of syngeneic N_3ph-derivatized spleen targets by *H-2^d* stimulated CTL*

Exp.	Responder	Stimulator	Targets†		
			C57BL/10	C57BL/10-N_3ph	B10.D2
I	C57BL/6 (*H-2^b*)	B10.D2 (*H-2^d*)	0	27	54
II	C57BL/6 (*H-2^b*)	DBA/2 (*H-2^d*)	0	31	N.T.‡
			B10.BR	B10.BR-N_3ph	B10.D2
I	B10.BR (*H-2^k*)	DBA/2 (*H-2^d*)	0	27	—

* Values are percent specific release of ^{51}Cr.
† Effector to target ratio was 40:1. Spontaneous release for C57BL/10 was 55%, 55% for B10.D2, and 35% for B10.Br.
‡ N.T., not tested.

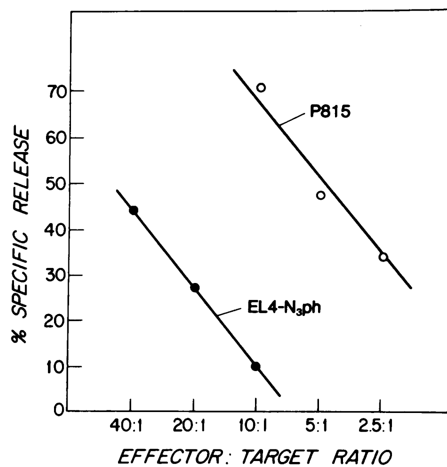

FIG. 1. Cytolysis of EL4-N3ph (*H-2*^{b}) and P815 (*H-2*^{d}) tumor targets by B6 (*H-2*^{b}) anti-B10.D2 (*H-2*^{d}) effector cells. The percent specific release of ^{51}Cr was determined for several effector to target ratios. Spontaneous release for EL4-N3ph was 40%, and for P815 20%.

ratios of B6 anti-DBA/2 effectors assayed on P815 and EL4-N3ph targets. The results give nearly parallel lines. By comparing equivalent percent specific release, we show that approximately 10 times as many effectors were required to attain the same specific release from EL4-N3ph as compared to P815.

Inhibition of the cytolysis of N3ph derivatized targets, with *H-2* loci identical to the effector, by cold tumor targets

The specificity of the CTL raised against allogeneic cells which are active on N3ph-conjugated syngeneic cells was investigated by using cold targets as inhibitors. Two types of effects were observed: (*i*) a highly significant inhibition caused by the addition of cold N3ph-syngeneic targets, and (*ii*) a weaker inhibition caused by nonconjugated tumor targets irrespective of whether they were syngeneic or allogeneic to the effector.

Fig. 2 demonstrates that cytolysis of ^{51}Cr-labeled EL4-N3ph

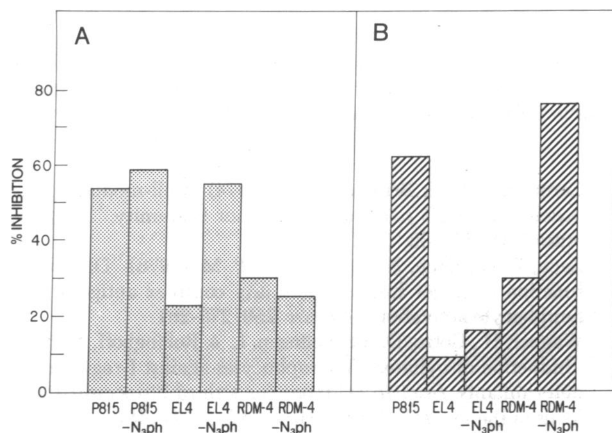

FIG. 2. Inhibition of cytolysis by competition with nonradiolabeled tumor targets. (*A*) B6 (*H-2*^{b}) anti-B10.D2 (*H-2*^{d}) effectors were assayed at an effector/target ratio of 20:1 on ^{51}Cr-labeled EL4-N3ph (*H-2*^{b}). Specific release was 55% in the absence of nonradiolabeled targets. (*B*) B10.BR (*H-2*^{k}) anti-B10.D2 (*H-2*^{d}) effectors were assayed at an effector/target ratio of 40:1 on ^{51}Cr-labeled RDM-4-N3ph (*H-2*^{k}). Specific release was 30% in the absence of nonradiolabeled targets. All nonradiolabeled targets were added at a ratio of 40 to 1 ^{51}Cr-labeled target. Spontaneous release for EL4-N3ph was 35%, and for RDM-4 N3ph 10%.

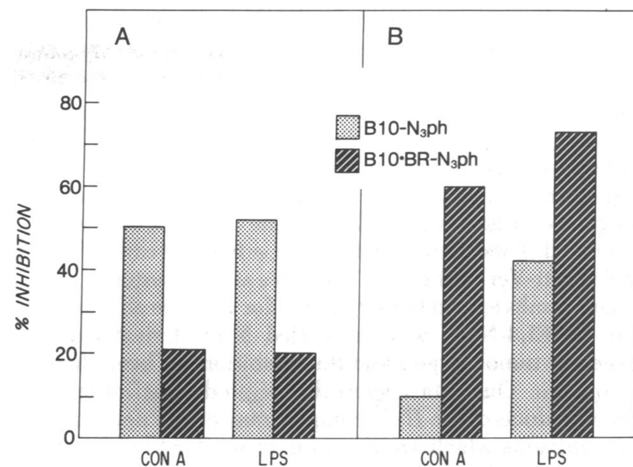

FIG. 3. Inhibition of cytolysis by competition with nonradiolabeled concanavalin A (Con A) or lipopolysaccharide (LPS)-induced spleen lymphoblasts. (*A*) B6 (*H-2*^{b}) anti-B10.D2 (*H-2*^{d}) effectors were assayed at an effector/target ratio of 40:1 on ^{51}Cr-labeled EL4-N3ph (*H-2*^{b}). Specific release was 45% in the absence of nonradiolabeled targets. (*B*) B10.BR (*H-2*^{k}) anti-B10.D2 (*H-2*^{d}) effectors were assayed at an effector/target ratio of 40:1 on ^{51}Cr-labeled RDM-4-N3ph (*H-2*^{k}). Specific release was 37% in the absence of nonradiolabeled targets. All nonradiolabeled targets were added at a ratio of 40 to 1 ^{51}Cr-labeled target. Spontaneous release for EL4-N3ph was 30%, and for RDM-4-N3ph 10%.

by B6 anti-DBA/2 effector cells was inhibited most effectively by the addition to the assay of nonradiolabeled P815 tumor cells syngeneic to the stimulator or EL4-N3ph tumor cells syngeneic to the targets. Unlabeled EL4 and unrelated RDM-4 or RDM-4-N3ph were far less efficient in inhibiting lysis. When B6 anti-DBA/2 effector cell lysis of ^{51}Cr-labeled P815 was studied, inhibition by unlabeled P815 but not by unlabeled EL4-N3ph was observed (data not shown). ^{51}Cr RDM-4-N3ph lysis by B10.BR anti-DBA/2 effectors was inhibited most effectively by unlabeled P815 and RDM-4-N3ph. Thus, in a situation where the ^{51}Cr target is RDM-4-N3ph, RDM-4-N3ph cold targets are able to inhibit lysis to a greater extent than EL4-N3ph.

These results indicate that: (*i*) the CTL mediating lysis of EL4-N3ph belong to a subset of CTL generated by alloimmunization with DBA/2; and (*ii*) these CTL interact specifically with the EL4-N3ph targets. The ability of EL4-N3ph cold targets to inhibit lysis far more effectively than RDM-4 N3ph cold targets excludes the possibility that N3ph modification has resulted in an immunologically nonspecific interaction between effector and target cells. In data not shown, it was also demonstrated that the small degree of nonspecific inhibition by F9, a teratocarcinoma lacking *H-2* serological determinants was not increased by N3ph derivatization.

Inhibition of cytolysis by cold spleen targets

To demonstrate that the effector-target interaction was specific for the gene products of the H-2 complex of the target, we utilized cold target lymphoblasts from congenic resistant strains with the same B10 genetic background. As shown in Fig. 3, cytolysis of ^{51}Cr-labeled EL4-N3ph by B6 anti-B10.D2 effectors was inhibited much more effectively by unlabeled concanavalin A or lipopolysaccharide-induced B10-N3ph lymphoblasts than by B10.BR-N3ph lymphoblasts. Conversely, lysis of ^{51}Cr RDM-4-N3ph by B10.BR anti-B10.D2 effectors was inhibited to a considerably greater extent by unlabeled B10.BR-N3ph lymphoblasts than by B10-N3ph lymphoblasts.

DISCUSSION

Normal spleen cells cocultured with irradiated allogeneic stimulator spleen cells develop cytolytic effector cells able to lyse syngeneic N_3ph-derivatized tumor or spleen targets in addition to the allogeneic stimulator cells against which they have been raised. Competitive inhibition with cold targets demonstrated that the CTL population effective on modified syngeneic targets was a subset of the population of all CTL generated. These results confirm and extend the earlier findings of Schmitt-Verhulst *et al.* (7, 12) in a similar system.

Our results extend those reported for we were able to inhibit lysis of EL4-N_3ph with unlabeled N_3ph-derivatized $H\text{-}2^b$ spleen or tumor targets and the inhibition has been shown to be specific. Our data suggest that N_3ph derivatization of the gene products of the $H\text{-}2^b$ complex may create new antigenic determinants which are the same or similar to native determinants coded for by the $H\text{-}2^d$ complex. N_3ph derivatization of the $H\text{-}2^k$ complex may also create new antigenic determinants which are the same or similar to determinants coded for by the $H\text{-}2^d$ complex, but some of these new determinants must be different from those created by N_3ph modification of the $H\text{-}2^b$ gene products.

We are aware that these findings can also be explained by a dual receptor model in which the receptors for self MHC products bind crossreactively to allogeneic MHC products. If, however, the second antigen receptor specific for N_3ph is clonally distributed on the CTL, then our data that 10% of all CTL raised to an unmodified allogeneic stimulator lyse N_3ph-syngeneic cells is not easily accounted for by such a model.

It should be noted that allogeneically-sensitized effectors were able to lyse N_3ph-modified targets that did not have *H-2* loci identical to the effector or the stimulator (data not shown). The extensive crossreactive cytolysis of unmodified targets without H-2 *loci* identical to the effector or stimulator made it difficult to evaluate the role of N_3ph derivatization. Furthermore, we have observed some lysis of unmodified tumor targets with H-2 loci identical to the effector cells (Table 1) which could conceivably be due to tumor-associated antigens that are the same or similar to $H\text{-}2^d$ antigens (13). Similar results have been reported recently by Sondel *et al.* in a human system (14).

As mentioned earlier, we have previously reported that although effector cells generated to N_3ph-derivatized syngeneic spleen cells lyse N_3ph-derivatized target cells that have H-2 loci identical to the stimulator most effectively, they also lyse N_3ph-derivatized allogeneic targets. Antisera against the gene products of the *H-2* complex was shown to inhibit this crossreactive lysis of the N_3ph-derivatized allogeneic targets, and minimal lysis was demonstrated on unmodified allogeneic targets. Thus, when effector cells were generated to N_3ph-derivatized syngeneic stimulators, the majority of the CTL generated were to new antigenic determinants that were *not* necessarily the same or similar to gene products of the conventional *H-2* haplotypes.

Therefore, from our studies of the specificity of CTL cytolysis of N_3ph-derivatized syngeneic and allogeneic targets, it appears that: (*i*) CTL generated to N_3ph-modified syngeneic stimulators are able to crossreactively lyse N_3ph-modified allogeneic targets and (*ii*) CTL generated to unmodified allogeneic spleen cells are able to crossreactively lyse N_3ph-modified targets that are syngeneic to the effector.

These data reflect the extensive crossreactivity which exist between cytolytic T cells specific for allogeneic MHC products and modified syngeneic and modified allogeneic MHC molecules. In addition, our results and those of others indicate that allogeneically-stimulated CTL are able to lyse syngeneic tumor targets bearing TSTA due probably to the nature of TSTA as modified products of the MHC (13, 14).

Because CTL are restricted to products of the MHC in allogeneic, xenogeneic, or modified syngeneic form, the results of this study may be interpreted as evidence that the function of CTL is the detection of alterations from self on these critical MHC molecules as a result of viral infection, chemical modification, or malignant transformation. The MHC product on the cells of an allogeneic individual may be viewed as a homogeneous variant of modified autologous counterparts against which there exist numerous CTL clones. From the point of view of crossreactivity, a similar situation was encountered by one of us (B. Benacerraf) when an experiment model for the production of human rheumatoid factor was proposed. Immunization of rabbits with autologous gamma globulins deliberately denatured in various ways stimulated the formation of antibodies able to react best with native foreign gamma globulins (15).

If, as we propose, the reactivity of T cells for allogeneic and xenogeneic MHC products can be considered as an example of cell populations reactive with modified autologous products of the MHC, and if these cells use V_H regions as antigen receptors as indicated by the recent studies of Binz and Wigzell (16), how can the restrictions of T cells to MHC products be achieved? Jerne proposed earlier a model for the generation of diverse immunoglobulin receptors for all specificities (17) which is more appropriate in our opinion to the commitment of T cells to MHC products and their variants. According to this modified model, T cells in the thymus, reactive to autologous MHC products, would be stimulated to proliferate and mutate the genes coding for their receptors so that only those cells with receptors no longer reactive to autologous MHC products, but reactive to altered MHC products, could be selected to migrate to peripheral lymphoid organs. This process should result in a large population of T cells specific for altered MHC products as achieved in our experiments. This model would also predict that immature T cells in the thymus would be potentially autoreactive if functionally mature and that malignant clones from these cells may express autoimmune reactivities to autologous products of the MHC.

This work was supported by National Institutes of Health Grants CA-14723 and CA-09130 and by a Public Health Service International Research Fellowship of the National Institutes of Health.

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