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H-2 I Alloantigens and Recall of Memory Cytotoxic Responses

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AQR mice were immunized with H-2K and H-2 I encoded alloantigens presented by (Ax6R)F_1 splenocytes. Spleen cells from these alloimmune mice were subsequently restimulated in vitro with B10.A lymphocytes and/or B10.T(6R) lymphocytes, thus presenting them with the immunizing H-2K and H-2 I alloantigens independently. When stimulated with B10.A lymphocytes, alloimmune lymphocytes develop significant cytotoxicity against the immunizing H-2K target antigens. When stimulated with a similar number of B10.T(6R) spleen cells, alloimmune lymphocytes undergo a prominant proliferative response, but develop little, if any, cytotoxicity against the immunizing H-2 K target antigens. The most efficient restimulation of cytotoxicity occurs when the alloimmune spleen cells are simultaneously restimulated by B10.A and B10.T(6R) lymphocytes. Stimulation with the immunizing H-2 I alloantigens alone is not sufficient for regeneration of detectable cytotoxic responses from alloimmune spleen populations. Stimulation with the immunizing H-2K alloantigens alone appears to be both necessary and sufficient to stimulate alloimmune cytotoxic responses. Although the immunizing H-2 I alloantigens are apparently not required to generate alloimmune cytotoxic responses, they markedly potentiate the cytotoxic responses induced by the immunizing H-2K alloantigens.

Key words: H-2K alloantigens, mixed lymphocyte culture, H-2 I alloantigens, cytotoxic memory, alloimmune responses

Naive lymphocyte populations generate cytotoxicity when stimulated by cytotoxicity-defined (CD) alloantigens (cytotoxic target determinants encoded primarily by H-2K and H-2 D/L genes) in the presence of lymphocyte-defined (LD) alloantigens (proliferative stimuli associated primarily with H-2 I and Mls gene products). Isolated CD alloantigens can be presented by UV-irradiated or heat-treated allogeneic spleen cells, and by irradiated H-2K disparate thymocytes. Cells thus selected or treated induce no detectable proliferation or cytotoxic activity. If accompanied by a strong LD stimulus, such as H-2 I disparate spleen cells, CD alloantigens induce high levels of antigen-specific

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cytotoxic activity. CD alloantigens accompanied by a weak LD stimulus induce less prominent cytotoxic activity. For example, H-2K disparate spleen cells stimulate weak proliferative responses, thus they display H-2K CD antigens in the context of a weak LD activity. Unlike H-2K disparate thymocytes, H-2K disparate spleen cells induce low but detectable levels of primary cytotoxic activity, presumably due to the additional proliferative stimulus present. Thus, under defined mixed lymphocyte culture conditions, both CD and LD alloantigens are required to induce a detectable primary cytotoxic response (for review, see [1]).

In contrast, lymphocytes previously sensitized in vivo to H-2K or H-2 D/L (CD) alloantigens can reexpress cytotoxicity restimulated in vitro only by the immunizing CD alloantigens, as displayed by UV-treated lymphocytes [2]. The effect of LD alloantigen restimulation of alloimmune cells has not been clearly demonstrated. It has been reported that alloimmune spleen cells stimulated by third-party lymphocytes unrelated to those used for in vivo priming develop cytotoxicity directed at the immunizing target determinants [3], perhaps because the LD activity of the third-party cells is capable of activating alloimmune cytotoxic cells. Furthermore, it has been reported that in vitro sensitized cytotoxic cells can be reactivated better by cells presenting LD activity than by cells presenting only the immunizing CD antigens [4]. Thus, we wished to determine if LD alloantigens influence the development of cytotoxicity by in vivo sensitized lymphocytes. This paper describes the effect of restimulation in vitro with the immunizing H-2 I alloantigens on the generation of cytotoxic activity by alloimmune lymphocytes.

METHODS AND MATERIALS

Mice

Male and female 8–12-week-old mice bred in this laboratory were used in these experiments. The strains employed were B10.A (kkdd), B10.T(6R) (qqqd), and (B.10.AxB10.T(6R))F_1 referred to hereafter as (Ax6R)F_1.

Mixed Lymphocyte Culture Conditions and Assay for Cell Mediated Lympholysis

Mixed lymphocyte culture (MLC) and cell-mediated lympholysis (CML) conditions were as described earlier [5]. Media consisted of EHAA containing 0.6% autologous normal mouse serum and 50 μM 2-mercaptoethanol (EHAA complete media) [6]. Cytotoxic activity was quantitated as % specific lysis of ^{51}Cr-labeled, Con A-stimulated spleen cells:

$$\% \text{ specific lysis} = \frac{\text{CPM (experimental)} - \text{CPM (spontaneous release)}}{\text{CPM (total release)} - \text{CPM (spontaneous release)}}$$

Data presented is representative of at least three independent experimental observations.

MLC Proliferation

Before cytotoxic activity was assayed, an aliquot from each MLC was removed and distributed among four replicate microtiter (Linbro No. 76-011-05) wells (0.2 ml/well) containing ^{3}H-thymidine (1 μCi/well). After 6 hr of incubation the cultures were harvested, and incorporated ^{3}H-thymidine was quantitated on a β-counter.

Sensitized In Vivo

Sensitization of AQR mice was accomplished by intraperitoneal injection of 20×10^6 $(Ax6R)F_1$ splenocytes in 0.5 ml saline. The mice were reimmunized seven days later and then rested at least 30 days before they were used as donors of in vivo sensitized spleen cells.

Heat Treatment of MLC Stimulator Cells

This technique is a modification of the procedure described by Eijsvoogel et al [7]. Fifty million spleen or thymus cells were suspended in 5 ml Dulbecco's phosphate buffered saline and incubated in a 45°C water bath for 30 minutes. They were then washed, counted, and resuspended in EHAA complete medium before use as MLC stimulator cells.

RESULTS

These experiments were designed to show the effect of restimulation with immunizing H-2 I alloantigens on the in vitro recall of memory cytotoxic responses. Donors of memory cytotoxic lymphocytes were AQR mice immunized previously with H-2K and H-2 I alloantigens on $(Ax6R)F_1$ spleen cells. To demonstrate the presence of a memory cytotoxic population, we first asked if the immunizing H-2K alloantigens were sufficient to restimulate cytotoxic responses in alloimmune AQR spleen populations. B10.A thymocytes were used to present the immunizing CD alloantigens. Use of thymocytes in this manner was based on the observation that nonimmune lymphocyte populations do not proliferate or generate cytotoxic activity when stimulated by H-2K different thymocytes unless a third-party cell displaying LD activity [2] or soluble helper activity [8] is included in the MLC. Hence, by definition, H-2K region-dissimilar thymocytes present adequate CD alloantigens but no detectable allogeneic LD activity. The advantage of using H-2K different thymocytes to present CD alloantigens is that it is unnecessary to pretreat them with heat, UV-irradiation, or glutaraldehyde to remove functional LD activity [9].

We observed experimentally that alloimmune AQR spleen cells stimulated in vitro with B10.A thymocytes generate significant cytotoxic activity (Table I), even if the thymocytes are first heat-treated to further insure the lack of functional LD activity [7, 9]. Under similar conditions, B10.A thymocyte populations cannot induce cytotoxic activity in non-immunized AQR spleen cultures. Hence, AQR mice immunized with $(Ax6R)F_1$ spleen cells possess a population of memory cytotoxic lymphocytes. These memory lymphocytes can be distinguished from primary cytotoxic precursors by their ability to develop cytotoxic activity when stimulated in vitro by the immunizing CD alloantigens, which were presented in these experiments by means of B10.A thymocytes.

We next asked if the immunizing H-2 I alloantigens presented on B10.T(6R) spleen cells could similarly induce memory cytotoxic responses in the alloimmune spleen populations. We observed that alloimmune AQR spleen cells stimulated in vitro with B10.T(6R) spleen cells fail to generate detectable levels of cytotoxic activity against target cells displaying the immunizing H-2K alloantigens, even though a significant proliferative response is observed (Table II). These same alloimmune AQR cells readily generate significant cytotoxic activity against H-2K-bearing targets as early as day 3 of mixed lymphocyte

TABLE I. Ability of Immunizing CD Alloantigens to Reactivate Memory Cytotoxic Response

Responder population	Stimulator population	% Specific lysis αB19,A day 4 25:1	day 4 5:1	day 6 25:1	day 6 5:1
1. 10^7 2° AQR spl[a]	AQR spl_X	− 1.6	1.3	− 5.0	− 2.8
2. "	$(Ax6R)F_1$ spl_X	46.6	18.9	62.5	37.9
3. "	B10.A spl_X	29.0	8.6	48.4	27.0
4. "	B10.A $thym_X$	20.7	7.4	58.7	28.8
5. "	B10.A $thym_\Delta$	11.7	1.3	52.1	17.2
6. 10^7 1° AQR spl	AQR spl_X	2.6	N.D.	− 1.8	2.2
7. "	B10.A spl_X	1.2	3.3	31.6	7.2
8. "	B10.A $thym_X$	− 0.6	3.3	2.1	1.8
9. "	B10.A $thym_\Delta$	0.1	0.8	− 4.0	− 1.8

[a]2° AQR immunized with $(Ax6R)F_1$ spleen cells 30 days in advance. AQR (qkdd); B10.A (kkdd); B10.T(6R) (qqqd); x = 2000R; Δ = 45°C, 30 minutes.

TABLE II. Inability of Immunizing LD Alloantigens to Reactivate Memory Cytotoxic Responses

10^7 2° AQR spleen cells[a] stimulated with	Day 5 MLR CPM × 10^{-3}	% Specific lysis α(Ax6R)F[1] day 3 40:1	day 3 20:1	day 5 20:1	day 5 5:1
1. No stimulators	1.3	− 0.9	3.5	− 8.5	− 8.2
2. 10^7 B10.T(6R) spl_X	13.9	− 1.0	− 2.2	1.2	0.9
3. 10^6 "	5.5	− 1.6	− 1.9	−11.5	− 8.4
4. 10^5 "	1.0	0.9	− 0.8	−20.7	−10.9
5. 10^7 B10.A spl_X	6.3	6.9	3.4	21.5	7.1
6. 10^6 "	0.9	10.2	6.1	− 2.7	− 6.8
7. 10^5 "	0.4	− 1.4	0.1	−14.2	ND[b]
8. 10^7 $(Ax6R)F_1$ spl_X	14.6	24.2	13.8	47.6	33.2
9. 10^6 "	6.4	21.1	18.1	35.5	21.5
10. 10^5 "	1.3	− 1.1	− 3.2	− 8.4	− 3.8

[a]Donors immunized with $(Ax6R)F_1$ spleen cells 30 days in advance. AQR (qkdd); B10.A (kkdd); B10.T(6R) (qqqd); (x) = 2000R.
[b]ND, not done.

culture when stimulated with $(Ax6R)F_1$ spleen cells or B10.A spleen cells. Hence, the immunizing LD alloantigens, as presented by H-2 I dissimilar spleen cells, are inefficient inducers of memory cytotoxic responses.

Finally, we asked if the presence of the immunizing H-2 I alloantigens has any influence on the memory cytotoxic response induced by immunizing H-2K alloantigens. When alloimmune AQR spleen cells are stimulated with both B10.A and B10.T(6R) spleen cells, highly significant cytotoxicity is generated (Table III). Such cytotoxicity is comparable to levels induced by the immunogen, $(Ax6R)F_1$ spleen cells, and is significantly higher than the cytotoxicity induced by either H-2K or H-2 I alloantigen stimulation

TABLE III. Immunizing LD Alloantigens Potentiate CD-Induced Reactivation of Memory Cytotoxic Responses

Responder population	Stimulator population	Day 5 MLR CPM × 10^{-3}	Day 5% specific lysis α(Ax6R)F_1 20:1	5:1
1. 2° AQR spl[a]	none	0.8	– 3.8	– 2.5
2. "	10^7 (Ax6R)F_1 spl_X	15.8	57.5	47.1
3. "	5×10^6 (Ax6R)F_1 spl_X	19.5	59.6	41.6
4. "	5×10^6 B10.A spl_X	4.2	18.3	13.5
5. "	5×10^6 B10.T(6R) spl_X	11.9	3.9	0.2
6. "	5×10^6 B10.A spl_X + 5×10^6 B10.T(6R) spl_X	16.6	55.4	30.3
7. 1° AQR spl	none	0.3	– 1.7	0.0
8. "	10^7 (Ax6R)F_1 spl_X	10.7	24.0	4.3
9. "	10^7 B10.A spl_X	4.1	1.3	0.1
10. "	10^7 B10.T(6R) spl_X	8.8	– 7.3	– 4.3

[a]Donors immunized with (Ax6R)F_1 spleen cells 30 days in advance. AQR (qkdd); B10.A (kkdd); B10.T(6R) (qqqd); x = 2000R.

alone. Hence, co-stimulation with immunizing LD (H-2 I) alloantigens potentiates cytotoxic response induced in alloimmune spleen populations by the immunizing CD (H-2K) alloantigens.

DISCUSSION

These experiments describe the influence of the immunizing LD alloantigens on the in vitro recall of memory cytotoxic responses from in vivo sensitized splenocyte populations. We observed that 1) immunizing CD alloantigens, in the form of H-2K different thymocytes, were sufficient to induce memory cytotoxic responses directed at the immunizing H-2K alloantigens; 2) immunizing LD alloantigens, in the form of H-2I different splenocytes, were not sufficient to induce detectable cytotoxic responses; and 3) stimulation with immunizing LD alloantigens potentiates the in vitro cytotoxic response induced by the immunizing CD alloantigens.

These experiments further confirm that alloimmune spleen populations contain a memory cytotoxic subpopulation, defined by the ability or those cells to develop cytotoxicity in response to "isolated" immunizing CD alloantigens. Non-immunized lymphocyte populations do not develop cytotoxicity under similar conditions unless CD alloantigens are accompanied by an LD stimulus such as H-2 I dissimilar lymphocytes. These observations are in agreement with those of others, who were also able to restimulate cytotoxicity in alloimmune lymphocytes with "isolated" CD antigens in the form of UV-treated lymphocytes [2, 9], heat-treated lymphocytes, glutaraldehyde-fixed lymphocytes [9], or crude membrane fragments [10, 11]. Our studies further show that this list of available techniques for presentation of "isolated" CD alloantigens should include presentation via H-2K different thymocytes.

Earlier studies from this laboratory showed that a memory cytotoxic population develops in mice immunized only with H-2K alloantigens [2]. Our studies employed alloimmune lymphocytes derived from donors immunized simultaneously with H-2K and

H-2 I alloantigens. Since donors immunized with either protocol develop spleen populations which generate cytotoxicity in response to the immunizing CD alloantigens alone, we conclude that a memory population develops in vivo following immunization with H-2K (CD) alloantigens, whether or not they are accompanied by H-2 I (LD) alloantigens. Although H-2 I alloantigens do not appear to be required to establish a memory cytotoxic cell population in vivo, we do not rule out the possibility that the H-2 I antigens may influence the size of the memory cytotoxic cell pool.

In these experiments we observed that the prominant cytotoxic activity associated with memory cytotoxic responses cannot be induced by the immunizing LD alloantigens in the form of H-2 I different spleen cells, although the LD alloantigens do stimulate strong proliferative responses in alloimmune spleen populations. LD stimulation has several effects on nonimmunized lymphocytes, one of which is to induce production of nonspecific soluble activities which potentiate weak primary cytotoxic responses [8]. Data will be presented elsewhere that alloimmune populations also respond to an LD stimulus by producing soluble helper activity (manuscript in preparation). Others have reported that soluble helper activity induced from immune cell populations is capable of inducing cytotoxicity in alloimmune spleen populations in the absence of allogeneic stimulator cells [12]. Together these observations suggest that alloimmune lymphocytes should generate soluble helper activity when stimulated with the immunizing LD antigens and that memory cytotoxic responses should result. It is not clear why this was not observed under these conditions and we are continuing investigations in this area.

It has been reported that third-party lymphocytes unrelated to those used for in vivo sensitization can induce cytotoxicity from alloimmune spleen cells [3]. The author suggests that either cross-reactive alloantigens displayed by the third-party cells can induce a secondary cytotoxic response or that alloantigens displayed by the third-party cells induce nonspecific soluble signals which, in turn, activate alloimmune cytotoxic cells. Since we are unable to stimulate significant cytotoxicity in alloimmune spleen populations with the immunizing H-2 I alloantigens, despite the fact that such stimulation clearly induces proliferation (Table II) and production of nonspecific soluble helper activity (unpublished observations), we favor the first of these two interpretations.

Our observation that under similar conditions significant cytotoxicity is readily induced in in vivo sensitized spleen populations by stimulation with the immunizing H-2K antigens but poorly induced by stimulation with the immunizing H-2 I antigens is in obvious contrast with published information on induction of cytotoxicity in spleen populations sensitized in vitro. Spleen cells recovered from 14-day MLCs rapidly reexpress cytotoxicity following stimulation with H-2 I or Mls (LD) alloantigens, but not the sensitizing H-2K or D (CD) alloantigens presented by UV-irradiated spleen cells [4]. This striking difference in responsiveness to CD or LD alloantigen stimulation suggests that not only do in vivo and in vitro sensitized lymphocytes differ from nonsensitized lymphocytes, but also from each other.

Despite the fact that the immunizing LD alloantigens are very inefficient at inducing alloimmune cytotoxic responses, they appear to potentiate such responses induced by the immunizing CD alloantigens. The question still remains as to how the augmentation of alloimmune cytotoxity responses is accomplished. One possible explanation is that LD-responsive cells act directly to expand the CD-responsive cytotoxic population. An alternative explanation is that the CD-induced cytotoxic pool is augmented by a primary cytotoxic pool induced by the simultaneous presence of LD and CD stimuli. Experiments are planned to determine if either or both of these mechanisms are operative.

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REFERENCES

1. Bach F, Bach M, Sondel P: Nature 259:273, 1976.
2. Kuperman O, Bach F: Scand J Immunol 6:161, 1977.
3. Bonavida B: In Sercarz E, Herzenberg L (eds): "Immune System: Genetics and Regulation." New York: Academic Press, 1977, pp 643–646.
4. Grillot-Courvalin C, Alter B, Bach F: J Immunol 119:1253, 1977.
5. Orosz C, Bach F: J Immunol 123:1419, 1979.
6. Peck A, Bach F: Scand J Immunol 4:53, 1975.
7. Eijsvoogel V, duBois M, Meinesz A, Bierhorst-Eijlander B, Zeylemaker W, Schellekens P: Transplant Proc 5:1675, 1973.
8. Scott J, Ponzio N, Orosz C, Finke J: J Immunol (in press).
9. Rollinghoff J, Wagner H: Eur J Immunol 5:875, 1975.
10. Engers H, Thomas K, Cerottini J-C, Brunner K: J Immunol 115:356, 1975.
11. Fast L, Fan D: J Immunol 120:1092, 1978.
12. Ryser J-E, Cerottini J-C, Brunner K: J Immunol 120:370, 1978.