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Antigen-Presenting Cells that Induce Anti-H-2K T-Cell Responses: Differences in Stimulator-Cell Requirements for Induction of Proliferation and Cell-Mediated Lympholysis

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Abstract. Splenic T or B cells, which have been depleted of adherent cells by passage through Sephadex G10 columns, fail to stimulate allogeneic lymph-node cells (LN) in primary mixed lymphocyte reactions (MLR) both when the stimulating antigens are H-2 plus Ia and H-2K only. This failure cannot be ascribed to lack of viability of G10-passed cells, since by dye exclusion they are 95 percent viable and can be induced to proliferate in vitro by exposure to LPS or allogeneic cells. Stimulation of MLR activity could be restored by addition of small numbers of plastic-adherent spleen cells (SAC) which had to be syngeneic with the G10-passed stimulator cells. Further, SAC alone without G10-passed cells induced MLR activity which was, on a cell-for-cell basis, 40 times more effective than that induced by unfractionated spleen cells. If the SAC were first depleted of Ia^+ cells, no stimulation was obtained. This result was observed both in cases where responder and stimulator strains differed across the entire *H-2*-gene complex and in a mutant-wild type combination (CBA and H-2^{km1}) in which the difference between the two strains has been mapped to the *K* region only. These results indicate that Ia^+ SAC contain a subset(s) of cells which are responsible for stimulation in MLR, regardless of whether the alloantigenic differences involve either Ia or H-2K. In contrast to the inability of G10-passed splenic cells to stimulate MLR activity, these cells were able to stimulate CTL from cytotoxic T lymphocyte precursors (CTL.P) in combinations where the antigenic differences between responder and stimulator were at the entire *H-2* complex or at *H-2K* only. However, SAC were more potent stimulators of cell-mediated lympholysis (CML) activity on a cell-for-cell basis. Thus, either CTL.P can be stimulated by nonadherent spleen cells or they are specifically sensitive to a small subpopulation of contaminating cells that cannot readily be removed by G10 passage.

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Introduction

The mixed leukocyte reaction (MLR) is an in vitro proliferative response of thymus-derived (T) lymphocytes against membrane-bound alloantigens. The alloantigens recognized can be encoded either by genes in the major histocompatibility complex (MHC) (Dutton 1966) or by genes outside of the MHC such as *Mls* (Festenstein et al. 1971, 1972). Within the MHC, MLRs are directed against antigens encoded by genes in the *K* region, the *D* region, and the *I* region (Klein 1975).

One of the questions that has been addressed in characterizing the MLR is the identification of the cell population(s) responsible for stimulating the response. Initially T cells (McLaurin 1972) and then B cells (Plate and McKenzie 1973) were thought to be stimulators of MLR activity. Later reports indicated that the MLR was induced by macrophages (Greineder and Rosenthal 1975). More recently, the antigen-presenting cells in the MLR have been described as lymphoid dendritic cells (Steinman and Witmer 1978), Langerhans cells (Stingl et al. 1978) and splenic adherent cells (SAC) (Ahmann et al. 1979). This latter population (SAC) is heterogenous so that the cell types contained within it that possess MLR-stimulating activity have yet to be identified. Despite this limitation two important points emerged from these recent studies. First, the MLR against Ia antigens requires the stimulating or antigen-presenting cell to be Ia-positive. Second, not all Ia-positive cells have the ability to stimulate an MLR, e. g., B cells (Ahman et al. 1979).

A large number of mouse strains are available that have undergone mutation in only their *H-2K* or *H-2D* genes. In many of these strains, the H-2K or D molecules have been well characterized biochemically, serologically, and functionally. For example, functionally a mutation in *H-2K* or *D* leads not only to sensitization of cytotoxic T cells (CTL) between the mutant strain and the strain from which the mutant arose, but also induces strong MLR (Forman and Klein 1975, McKenzie et al. 1977). Accordingly, most MLRs studied between an *H-2* mutant and the strain of origin represent responses against either H-2K or D antigens. Since these antigens are expressed on most somatic cells of the mouse, we wished to determine whether all splenic cells are capable of stimulating an anti-H-2K or -D MLR in a mutant-vs.-parental strain combination. Second, if we found that only a limited subpopulation of splenic cells can stimulate this MLR, we wished to ascertain whether the stimulating cell was required to be Ia-positive. The results of the experiments presented here demonstrate that the antigen-presenting cell in an anti-H-2K-directed MLR is found in Ia-positive splenic adherent cells but not in Ia-negative SACs or splenic B or T cells. When, however, the same fractionated cells were used to generate primary CTL, it was observed that SAC, G10-nonadherent and unfractionated spleen cells were all able to stimulate CML activity.

Materials and Methods

Mice. All mice used in these experiments were bred in our animal colony at the University of Texas Health Science Center at Dallas. A list of strains with their *H-2* genotypes is given in Table 1.

Table 1. *H-2* genotype of mouse strains

	K	*I-A*	*I-B*	*I-J*	*I-E*	*I-C*	*S*	*D*
C57BL/6, C57BL/10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
H-2^{bm1}	*bm1*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
CBA	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*
H-2^{km1}	*km1*	*k*	*k*	*k*	*k*	*k*	*k*	*k*
B10.Q	*q*	*q*	*q*	*q*	*q*	*q*	*q*	*q*
B10.S	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*
B10.S(7R)	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*d*

The mouse strains used in these experiments are listed and the alleles present at each of the subregions of the *H-2* gene complex are detailed.

Medium. Mixed lymphocyte cultures (MLC) were generated in RPMI 1640 supplemented with 1 percent normal mouse serum (NMS), penicillin/streptomycin/mycostatin, 2mM glutamine and 5×10^{-5} M-2mercaptoethanol. Cytotoxic T lymphocytes (CTL) were cultured in the same medium as above, except that it contained 7.5 percent fetal calf serum (FCS) instead of NMS.

Cells. Lymph nodes (LN) and spleens were teased apart and washed twice in balanced salt solution (BSS), and kept in a pellet on ice until further culture or fractionation. Adherent-cell-depleted spleen cells were prepared by passage of whole spleen cells over a Sephadex G10 column as described by Ly and Mishell (1974), and Ahman and co-workers (1979). B cells were prepared from G10-passed cells by incubation with monoclonal anti-Thy 1.2 (New England Nuclear) and complement. T cells were prepared from G10-passed cells by negative selection on anti-immunoglobulin (Ig) coated plastic Petri dishes (affinity-purified goat anti-mouse Ig) according to the method of Wysocki and co-workers (1978).

Splenic adherent cells (SAC) were prepared in the following manner. Whole spleens, exclusive of capsule, were teased apart. After removal of suspended cells, the sedimented fraction was incubated in serum-free RPMI 1640 containing 5 mg of collagenase and 1 mg DNAase (Grand Island Biological Company) at 37° C for 15 min. After washing twice in BSS, the splenic material was resuspended in RPMI 1640 supplemented with 5 percent FCS (5 ml for each spleen) and incubated in 60 mm plastic Petri dishes (5 ml/dish) for 4 h at 37° C. The dishes were then rinsed thoroughly of non adherent cells using Hanks' balanced salt solution without calcium or magnesium (HBSS without Ca/Mg), and then incubated for an additional 10 min at 37° C in HBSS without Ca/Mg supplemented with 2% chicken serum (CS) and 2 mM EDTA (Artzt et al. 1973). Adherent cells were then removed from the plates by vigorous pipetting and washed three times in BSS. SAC were kept pelletted on ice until resuspended for cultures.

All cell preparations were examined for viability by phase microscopy or by evaluation of trypan blue dye exclusion.

Mixed lymphocyte cultures (MLC). Five hundred thousand LN cells and varying numbers of 3000 R irradiated stimulator cells were cultured in 0.2 ml of MLC medium in flat-bottomed 96 well microtest plates and incubated for 5 days at 37° C. On day 5, 1 uCi of ^{3}H-thymidine was added to each well and incorporation of isotope into DNA was assessed after 4–8 by liquid scintillation counting. Results are expressed as total CPM.

Cytotoxic T-lymphocyte assay. MLC were set up as above in round-bottomed microtest plates. After 5 days of culture, 2 000–4 000 ^{51}-Cr-labeled Con A lymphoblasts were added to each well and percentage of isotope release determined after 4–6 h by direct gamma counting. Results are expressed as net percent release: (percentage of isotope released from targets in the presence of immune cells–percentage of isotope released from targets in the presence of control cells).

It should be noted that MLC were cultured with NMS supplements while cytotoxic lymphocytes were generated with FCS supplements. These were chosen because each culture supplement optimized the respective responses. Thus, MLR cultures in the presence of FCS gave high background stimulation with relatively low immune stimulation. Use of NMS failed to allow for the generation of cytotoxic effector cells.

Determination of latex ingestion. For this assay, $1–5 \times 10^6$ cells were suspended in RPMI 1640 supplemented with 15 percent FCS and 0.2 percent Latex beads and incubated overnight in 37° C or at 4° C in small test tubes. After washing once in BSS, the culture was resuspended in 1 ml of BSS and overlaid on a 1 ml layer of FCS. After centrifugation (1 500 rpm for 8 min), cells formed pellets through the FCS while free latex remained at the interface. The cells were then collected and examined on glass slides using oil emersion microscopy. A minimum of 200 cells was counted and a cell was considered positive for latex ingestion if it had taken up at least five latex beads. Results are expressed as percentage of latex-positive cells. Cells incubated at 4° C failed to ingest latex in all experiments.

Determination of nonspecific esterase activity. Nonspecific esterase activity was determined for formalin-acetone fixed cells according to a modification of the method of Yam and co-workers (1971). In this method, the cell-associated (cytoplasmic) esterase releases naphthol from the substrate, α-naphthyl butyrate (Sigma Chemical Company, St. Louis, Missouri). The naphthol is then coupled by the diazonium salt, hexazotized pararosanilin, to form an insoluble red precipitate. Esterase-negative cells appear green due to the counter strain, methyl green. A cell was considered esterase-positive only if it was stained dark red, and negative if stained pale pink or green.

Antisera. Monoclonal anti-Thy 1.2 was purchased from New England Nuclear and was used to deplete T cells at a dilution of 1 : 50 in a two-step complement-mediated cytotoxicity procedure. Fluoresceinated rabbit anti-mouse Ig was purchased from Cappel Laboratories, Cochranville, Pennsylvania A.TH anti-A.TL serum was produced by weekly immunization of A.TH mice with 10^7 A.TL spleen cells (i. p.). Sera from positive bleedings were pooled and used at 1 : 8 for depletion of Ia^k-positive cells. The following antisera were the generous gift of Dr. Ellen Vitetta. (1) Monoclonal anti-I-A^k (Salk line H 11.5.2), (2) affinity-purified goat anti-mouse Ig, (3) fluoresceinated $F(ab)_2$ goat anti-mouse IgG, which was used as the developing sera in the indirect immunofluorescent analysis of Ia-positive cells.

Results

Characterization of fractionated spleen cells. These experiments were designed to test the ability of various fractionated spleen-cell subpopulations from strains with known genetic differences to stimulate a proliferative response in primary mixed lymphocyte culture. Since it was necessary to assess the effectiveness of our cell-isolation and purification procedures, we characterized each cell population with regard to cell-surface phenotype, presence of nonspecific esterase activity, and the ability to ingest latex particles. Data from two representative experiments are presented in Table 2. Passage of CBA spleen cells through G10 columns does not significantly alter the proportion of Ia^+ or Ig^+ cells. However, such passage does markedly reduce the proportion of cells (1) capable of ingesting latex particles and (2) staining heavily for esterase, both of which are markers for macrophages (Yam et al. 1971). The two experiments presented indicate the range of differences that we noted in mouse spleens with respect to the percentage of cells which can be characterized as macrophages, and range from 2–5 percent (exp. 2) to 11.6–16.5 percent (exp. 1) of the total spleen cells. Thus, the relative enrichment of macrophages in the SAC population, while usually four- to eightfold, results in SACs that contain between 9.7–17 percent and 44–47 percent macrophages. The percentage of Ig^+ cells in the SAC population was reduced by an average of 50 percent.

When G10-passed cells were further purified into B and T cells, more than 95 percent of B cells were Ig^+ (treated with anti-Thy 1.2 and C′) and 3 percent of T cells were Ig^+ (data not shown).

Table 2. Characterization of fractionated CBA spleen cells

		Ig^+(%)*	Ia^{k+}(%)†	$\alpha\gamma 5a^+$(%)†	$Latex^+$(%)	$Esterase^+$(%)
Exp. 1	Whole spleen	28.7	21.3	0/300‖	11.6	16.5
	G10 × 1‡	30.0	26.7	0.5	0.3	1.5
	G10 × 2‡	27.4	25.4	0.6	0.2	1.0
	SAC	8.9	26.5	0/300‖	44.0	47.0
Exp. 2	Whole Spleen	41.7	64.7	3.7	2.0	5.0
	G10 × 1	43.4	64.9	2.4	1.2	1.0
	G10 × 2	41.2	54.9	3.4	0.3	1.0
	SAC	29.3	53.6	2.8	9.7	17.0

† Fractionated spleen-cell populations were anlayzed by direct or indirect† immunofluorescence. Fluoresceinated goat-anti-mouse Ig was used for direct staining. Anti-Ia^k was pooled A.TH anti-A.TL serum, and was developed using a fluoresceinated $F(ab')_2$ goat anti-mouse IgG. Mouse anti-γ5a was used as a hyperimmune serum control and was negative in all experiments.

‡ Refers to one or two sequential passages of cells through G10 columns.

‖ Three hundred cells were counted but no fluorescence-positive cells were observed.

Inability of G10-passed cells to induce proliferation in MLR among H-2K-incompatible strains. In order to determine whether nonadherent cells could stimulate MLR activity, we assessed the effect of G10 passage on the ability of spleen cells to stimulate an MLR. This was done between strains differing only at *H-2K* and between strains which differ across the entire *H-2* complex. G10 passage eliminated the ability of spleen cells to induce proliferation in H-*2* allogeneic MLR (Fig. 1 a and 1 b), confirming the data of Ahmann and co-workers (1979). In addition, the B-cell-enriched populations, which should contain more Ia-positive cells relative to whole spleen (Unanue et al. 1974) induced no proliferation. Similar results were obtained when we examined MLR activity between $H\text{-}2^{bm1}$ and C57BL/6, an *H-2* mutant wild-type strain combination differing at *H-2K* only (Fig. 1 a) (Bailey et al. 1971, Brown and Nathenson 1977). Thus, even though in this strain combination the H-2K alloantigen should be present on all splenic cells, $H\text{-}2^{bm1}$ cells were unable to induce MLR in C57BL/6 LN cells after passage through G10 columns.

Reconstitution of stimulation in MLC by splenic adherent cells (SAC). Since G10 passage should remove cells necessary for both antigen presentation in MLR and for support of in vitro cultures, we attempted to reconstitute the response by addition of SAC to G10-passed cells. In these experiments, SAC were added to G10-passed cells such that they constituted 10 percent of the total input of whole spleen (stimulator) cells. Addition of 5×10^4 SAC to 5×10^5 G10-passed cells reconstituted the response induced in 5×10^5 B6 LN cells to levels that were as good as or better than those induced by unfractionated (whole) spleen cells (Fig. 2 a and 2 b). In order to rule out the possibility that SAC had a nonspecific culture-support function, rather than providing the actual stimulation in MLR, we added 5×10^4 B6 SAC to 5×10^5 B6 LN cells (Fig. 2 a and 2 b). While additional responder B6 SAC enhanced the B6 anti-$H\text{-}2^{bm1}$ and B6 anti-B10.Q response to both unfractionated spleen cells (S) and to G10-passed cells plus SAC, (G10 + A), it did not allow proliferation in the response to G10-passed cells alone.

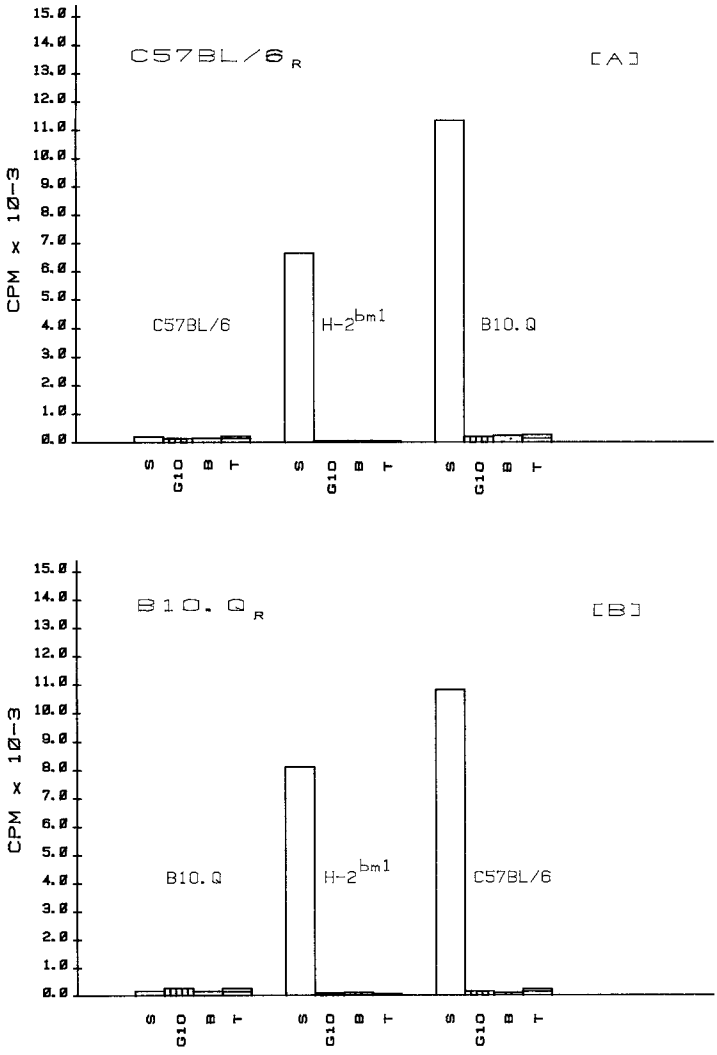

Fig. 1 A and B. 5×10^5 B6 LN cells were cultured for 5 days with 5×10^5 irradiated unfractionated spleen cells (S, □), G10-passed cells (G10, ▥), B cells (B, ⊡) or T cells (T, ▤) from the strains indicated. ^{3}H-Thymidine incorporation is given as total CPM $\times 10^{-3}$.

Thus, G10-passed cells are depleted of a required stimulator cell type(s), rather than being depleted of nonspecific accessory cells. Further, this result suggests that G10-passed cells do not exert a suppressive effect on the MLR.

The stimulation induced by SAC is not due to T cells copurifying with SAC. It is possible that adherent T cells, which copurify with SAC, could be responsible for MLR stimulatory activity, since T cells could induce proliferation in the LN cells by "back stimulation", or if antigen-activated, by release of soluble factors (Watson 1979). In order to test this possibility, SAC were pretreated with normal mouse

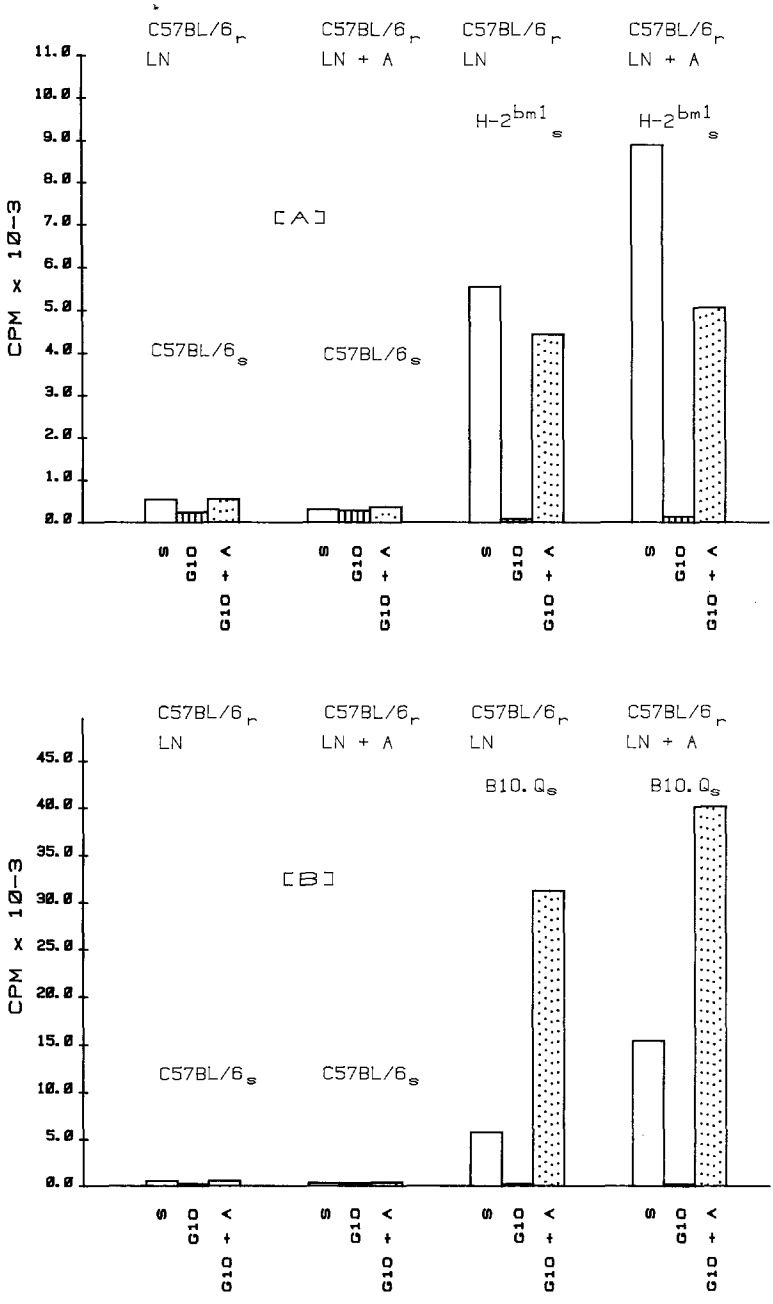

Fig. 2. 5×10^5 B6 LN cells or 5×10^5 B6 LN cells $+ 5 \times 10^4$ B6 SAC were incubated for 5 days with 5×10^5 irradiated spleen cells (S, □), 5×10^5 G10-passed cells (G10, ▥), or 5×10^5 G10-passed cells $+ 5 \times 10^4$ SAC (G10 + A, ⊡) for 5 days then assessed for ^{3}H-thymidine incorporation. Results are expressed at total CPM $\times 10^{-3}$.

serum plus guinea pig complement (NMS + C′) or with a monoclonal anti-Thy 1.2 plus C′. The results of this protocol (Fig. 3 a and 3 b) show that treatment of CBA or B10.Q SAC with anti-Thy 1.2 does not diminish their ability, either alone or together with 5×10^5 G10-passed cells, to stimulate MLR in H-2^{bm1} LN cells. The same antiserum eliminated the MLR and Con A response in unirradiated responding cells (data not shown). Thus, both completely *H-2*-incompatible (Fig. 3 b) and *H-2K*-only-incompatible (Fig. 3 a) MLR requires SAC as stimulator cells, and this stimulation is not due to T cells in this population.

Ia^+ SAC are required as stimulator cells in anti-H-2K MLR. It has been reported that in *H-2*-incompatible strains, Ia^+ SAC are the required stimulator cell type(s). However, since I region-encoded antigens are largely responsible for the proliferation induced in these combinations, (Bach et al. 1976), these results might simply reflect removal of antigen-positive cells. Thus, it is possible that if the response to non-Ia antigens were to be tested, Ia-negative SAC would prove stimulatory. In order to test this possibility, we examined the MLR between CBA and H-2^{km1}. The proliferative response in this combination maps to *H-2K* only (Blandova et al. 1975, Klein et al. 1975). SAC from H-2^{km1} and CBA were treated either with NMS + C′ or with one of two different anti-*I*-region reagents + C′. In the experiment presented in Figure 4 a, monoclonal anti-I-A^k (anti-I-A^k) was used, and in Figures 4 b and 4 c conventional A.TH anti-A.TL serum (anti-Ia^k) was employed. This latter serum should react with products of all *I* subregions of *H-2^k*. These experiments show that treatment of H-2^{km1} and CBA SAC with antisera against I-A^k or against Ia^k abrogates their ability to stimulate anti-H-2K MLR activity (Fig. 4 a and 4 b). This finding shows that I-A^k is expressed on antigen-presenting cells in MLRs directed against H-2K antigens. As a control, we also treated B10.S (7R) (Fig. 4 a) and B10.S (Fig. 4 b) stimulating cells with these same reagents, and no diminution of the allogeneic MLR was observed. Thus, MLR against *H-2K* only depends on Ia^+ SAC, as does the MLR against entire-*H-2*-incompatible strains (Fig. 4 c).

Titration of fractionated spleen cells as stimulators in MLR. In order to assess the degree of enrichment of cells capable of stimulating an MLR, we titrated in doubling dilutions whole spleen cells and SAC as stimulators in MLR. The results of one such experiment are given in Figure 5. Whole spleen cells fail to give MLR stimulation greater than twice the negative control value at input cell numbers of less than 62 500 cells. G10-passed cells fail to stimulate at any input cell number tested. On the other hand, SAC were stimulatory at even low input cell numbers, giving tenfold greater stimulation with less than 8000 stimulator cells. Although at high numbers of input stimulator cells SAC suppress the mixed lymphocyte reaction, by comparing the cell numbers of spleen and SAC required to produce equivalent thymidine incorporation at linear portions of the titration curves, we estimate that approximately 40-fold fewer SAC stimulate MLR activity on a cell-for-cell basis relative to whole spleen cells.

Titration of fractionated spleen cells as stimulators of cytotoxic T lymphocyte (CTL) activity. It has been reported by Petinelli and co-workers (1979) that G10-passed

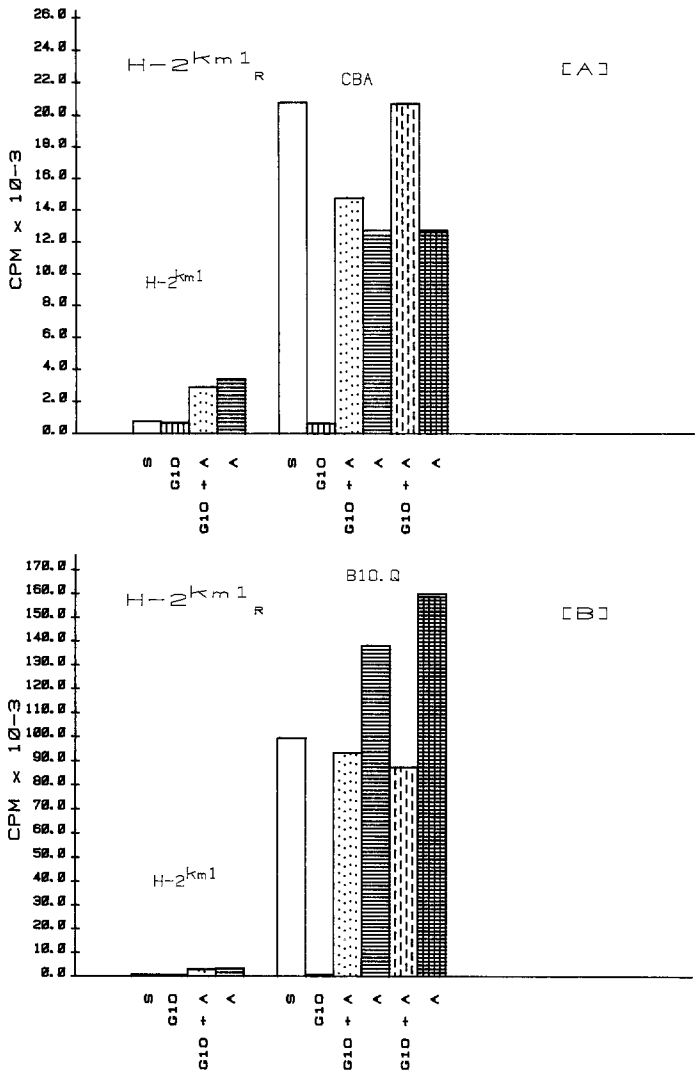

Fig. 3 A and B. B6 LN cells were cultured with irradiated spleen cells, G10-passed cells, or a mixture of G10-passed cells and 10^5 SAC. The SAC were treated either with NMS × C′ (G10 + A, ⊡; A, ▤) or with a monoclonal anti-Thy 1.2 + C′ (G10 + A, ▥; A, ▦). After 5 days the cultures were assessed for ^{3}H-thymidine incorporation.

cells are capable of stimulating CTL activity. In view of the differences observed in the ability of whole spleen, G10-nonadherent and SAC cells to stimulate MLR activity, we wished to determine if any differences existed between these cell populations with regard to their ability to induce CTLs in primary cultures. In the experiment presented in Figure 6, cells were passed through two sequential G10 columns to minimize the number of contaminating adherent cells present. When 22 222 or more stimulator cells were added to the MLCs, all three cell populations induced CTL activity to the same extent. However, as the input number of

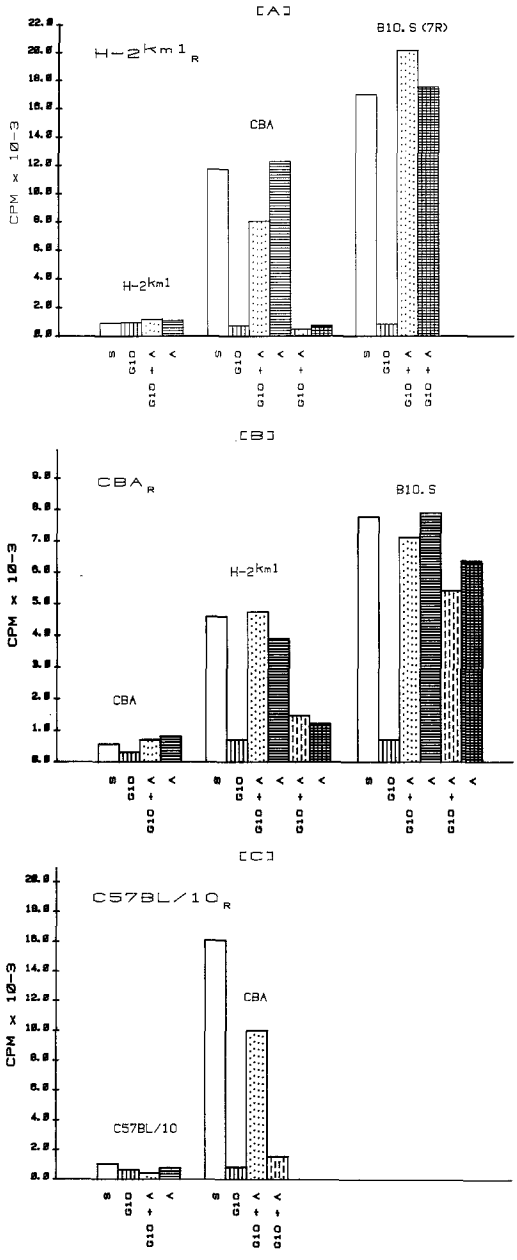

Fig. 4 A–C. Responding LN cells were cultured with irradiated spleen cells, G10-passed cells, or mixtures of G10-passed cells + 5×10^4 SAC for 5 days and assessed for ^{3}H-thymidine incorporation. The SAC were treated either with NMS + C′ (G10 + A ⊡, A ▤) or with antiserum directed against I-A^k (**A**) or against Ia^k (all subregions, **B** and **C**) (G10 + A ▦, A ⊞).

stimulator cells decreased, significant differences among the stimulator populations were observed. On a cell-for-cell basis, SAC are five times more stimulatory than whole spleen cells, which in turn are four times more stimulatory than G10-passed cells. This finding may indicate that MLR-reactive cells and CTLs have similar stimulator-cell requirements, but that CTL precursors are exquisitely sensitive to activation by small numbers of contaminating adherent cells whose activity may be

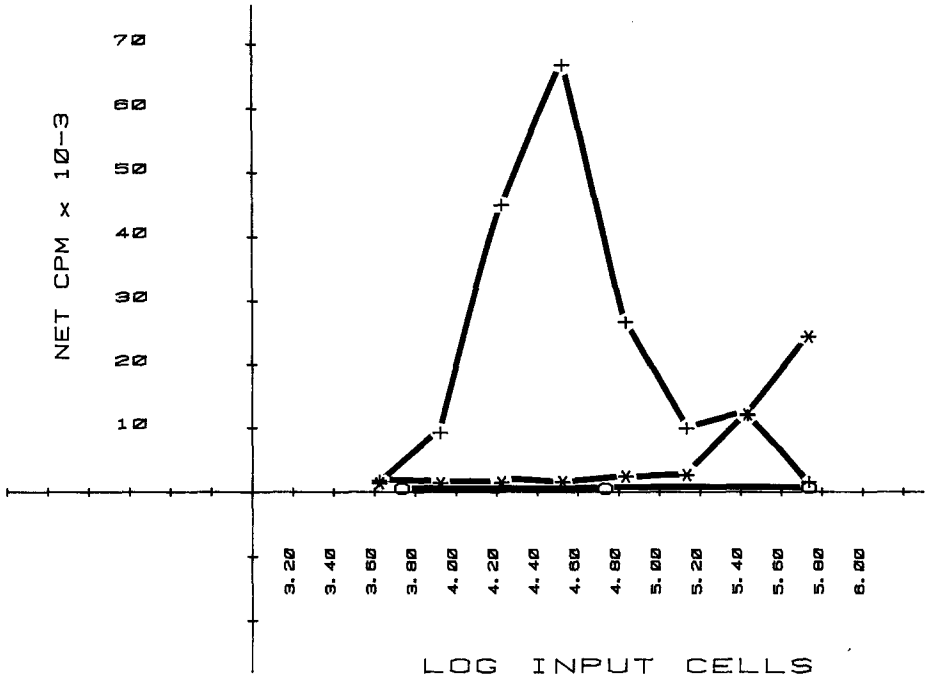

Fig. 5. 5×10^5 B6 LN cells were incubated with doubling dilutions of irradiated spleen cells (*) or SAC (+), or with 5×10^5, 5×10^4 and 5×10^3 G10-passed cells (○). Cultures were assessed for ^{3}H-thymidine incorporation after 5 days. Syngeneic stimulation is not shown in this figure but did not exceed 800 CPM. Results are expressed as total CPM $\times 10^{-3}$.

potentiated by the presence of fetal calf serum. Alternatively, CTL precursors may be activated by a number of different alloantigen-bearing stimulator cell types, although some stimulating cells may be quantiatively more effective in presenting antigen than others.

Discussion

The mixed lymphocyte reaction between *H-2*-incompatible strains involves the proliferation of T cells to specific alloantigens. The data of Widmer and co-workers (1973) demonstrated that, although most H-2-encoded antigens elicit mixed lymphocyte reactivity, antigens encoded by genes in the *I-A* subregion induced quantitatively the strongest response. Ahmann and co-workers (1979) recently showed that the antigen-presenting cell in anti-H-2 mixed lymphocyte reactions is an Ia-positive adherent cell. Further, Ia-positive nonadherent cells (e. g., B cells) are not able to stimulate mixed lymphocyte reactivity. While it was an unexpected finding that B cells could not induce MLR activity, it was not surprising to find that only those adherent cells that were Ia-positive could stimulate. Therefore, in this present study we examined lymphocyte reactions between *H-2K*-incompatible strains and showed that splenic adherent cells, but not splenic B or T cells stimulate

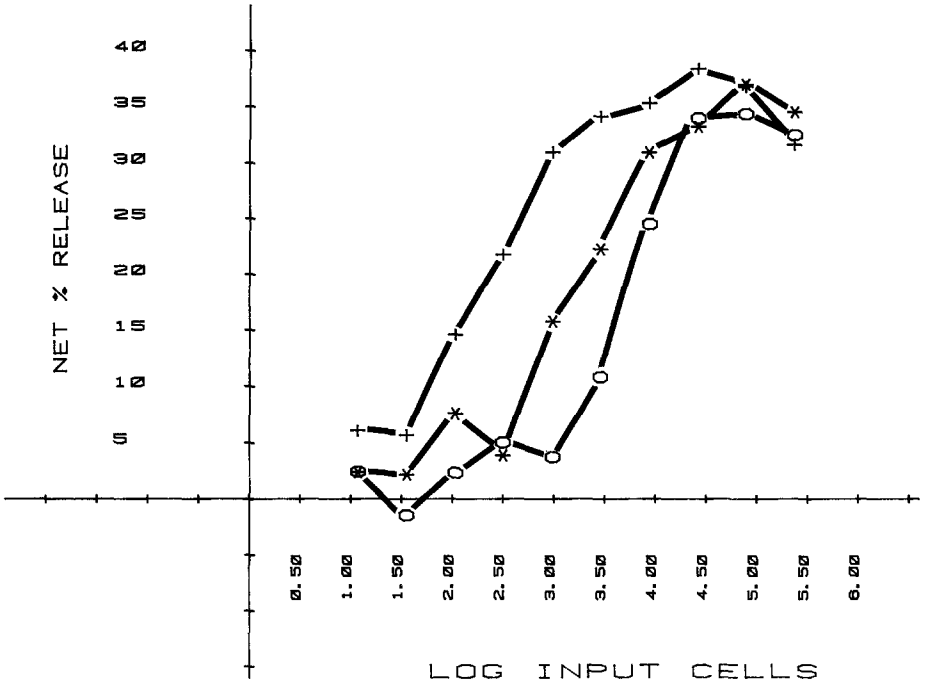

Fig. 6. 5×10^5 B6 LN cells were incubated for 5 days with tripling dilutions of irradiated spleen cells (*), SAC (+) or G10-passed cells (O) and assessed for CTL activity against ^{51}Cr-labeled Con A lymphoblasts. Results are expressed as net release.

this response. Further, although the antigen being recognized by the responder cells is encoded by *H-2K*, only Ia-positive splenic adherent cells could stimulate a response. This finding is in agreement with that of Minami and co-workers (1980).

The fact that Ia-positive cells are required to stimulate a mixed lymphocyte response directed against a non-Ia antigen, i. e., H-2K, raises the issue of whether the MLR against *H-2K* mutant strains is really directed against Ia- rather than H-2K-region antigens. In this regard, H-2 antigens from *H-2* mutant strains have been analyzed both serologically and biochemically. Results of such studies have revealed that *H-2K* mutant animals carry altered H-2K proteins (Ewald et al. 1979), consistent with the genetic mapping of the mutation (Blandova et al. 1975, Klein et al. 1975). No data have been obtained to indicate that Ia antigens have undergone structural or functional alterations (Klein 1975). Thus, serological, biochemical and genetic mapping studies indicate that the *H-2* mutant strains we have studied involve mutation at *H-2K* only.

The fact that Ia-positive splenic adherent cells stimulate MLR against either Ia or H-2K antigens, whereas other splenic subpopulations do not, suggests that the Ia phenotype serves as a marker for a differentiation state of this stimulator cell. Accordingly, cells able to present antigen to T cells may require some intrinsic physiological property which is only expressed at a certain differentiation stage. For example, antigen-presenting cells may need to supply an antigen-nonspecific signal to the T cell in order to trigger it. This signal could be in the form of an activating

factor, as has been described in other systems (Calderon et al. 1975, Unanue 1978, Watson 1979). Alternatively, the ability of a cell to present antigen to a T cell may involve having the proper density or arrangement of H-2 or Ia molecules. In this regard, the density of Ia antigens on a particular subpopulation of adherent cells has been shown to be much greater than that found on B cells. These cells, called lymphoid dendritic cells, not only have a high density of Ia antigens on their cell membranes, but have also been shown to be extremely potent stimulators of mixed lymphocyte reactions (Steinman and Witmer 1978). Whether these cells also have high densities of H-2K or D antigens has yet to be established.

An alternative explanation for the requirement of Ia-positive cells in presenting H-2K antigens to MLR-reactive T cells is that MLR-reactive cells are *H-2*-restricted by Ia antigens. In this case, the MLR-responding T cell against an H-2K alloantigen would recognize *H-2K* only in the context of *Ia*. Further, restimulation of primed anti-H-2K-reactive T cells would require that H-2K antigen be presented on stimulator cells bearing the same Ia antigens as the initial antigen-presenting cell. Possibly the antigenic unit which is recognized is an interaction antigen involving different cell-surface glycoproteins. A precedent for this hypothesis exists in the interaction of Ia molecules on the cell membrane. Thus, the beta (β) chain of *I-E/C* is encoded in the *I-A* subregion and forms an I-E/C dimer with an alpha (α) chain encoded by a gene in the *I-E/C* subregion (Jones et al. 1978, Cook et al. 1979). Serologically, hybrid determinants can be detected on I-E/C molecules formed from α and β chains from both parental haplotypes on F_1 heterozygous cells (Lafuse et al. 1980). In addition, Fathman and Hengartner (1980) have shown that individual clones of MLR-reactive T cells recognize an H-2 determinant on F_1 cells that is not found on either parental cell.

The hypothesis that recognition of H-2K or D antigens is restricted by Ia antigens could explain the findings of Zinkernagel and co-workers (1978) who observed that no vaccinia-virus-specific CTL could be generated from spleen cells of *I*-region-incompatible bone-marrow chimeras. They concluded that this failure was due to a requirement that the T helper cell interact with the same Ia antigen on both the virus-infected cell and on the CTL precursor cell. A similar conclusion was reached by von Boehmer and co-workers (1978) who studied CTL against male (Y) antigen in bone-marrow chimeras. However, an alternative explanation is that T helper cells recognize H-2K or D plus virus in the context of Ia antigens. Thus, the helper cells for the CTL precursors against virus or other extrinsic antigen(s) are restricted by both Ia and H-2K or D. While this might seem to require three receptors for the T helper cell, one specific for *H-2I*, one for *H-2K* or *D*, and one for virus or Y antigen, this may not be the case since CTLs raised against self-plus-virus or self-plus-minor H antigens can cross-react with H-2 allospecificities (Bevan 1977, Burakoff et al. 1978, Finberg et al. 1978). Therefore, some T helper cells recognizing mutant (allo) H-2K or D antigens in the context of Ia antigen(s) may also recognize virus-plus-H-2K/D in an Ia-restricted manner.

Although a subpopulation of splenic adherent cells stimulate MLR activity, the nature of this splenic adherent cell is unresolved. The adherent cells we tested are enriched for latex and esterase positivity, which are markers for mature macrophages (Yam et al. 1971); however, our data do not necessarily indicate that macrophages are the cells responsible for the stimulation. It is possible that a

dendritic cell, or some other type of adherent cell possesses the ability to stimulate a mixed lymphocyte reaction. Alternatively MLR activity may be stimulated by several cell types which differ from each other merely quantitatively.

Our present data indicate that H-2K antigens stimulate a mixed lymphocyte response only when presented on a subpopulation of Ia-positive cells. Data from other studies indicate that this phenomenon is not confined to anti-*H-2* responses. Thus, Cowing and co-workers (1978) have shown that T cells proliferating in vitro to protein antigens require that antigen be presented on Ia-positive splenic adherent cells. Similar results have been demonstrated in helper-T-cell assays (Singer et al. 1978, Niederhuber 1978). We have tested the ability of T cells to respond in secondary cultures following skin painting with contact sensitizing agents such as trinitrochlorobenzene (J. A. Trial and J. Forman, manuscript in preparation). In these circumstances we have also noted that only haptenated splenic adherent cells are able to present antigen. Thus, the presentation of antigen to proliferating and helper T cells apparently requires a similar or identical subpopulation of adherent cells to present antigen as is the case for the anti-H-2 MLR.

Petinelli and co-workers (1979) observed that splenic nonadherent cells could generate primary CTL effector cells. In the present study, we reinvestigated their finding and passed our antigen-presenting cells through two G10 columns to reduce the amount of contaminating adherent cells to a minimum. Such G10-passed cells were able to induce a CTL response against an H-2K mutant antigen. By titrating the number of splenic cells, splenic nonadherent cells, and splenic adherent cells required to induce CTL activity, we noted that splenic adherent cells were approximately five times more stimulatory than whole spleen cells, which were approximately four times more potent in inducing CTL activity than G10-passed cells. Further, as few as 100 splenic adherent cells were able to induce a CTL response when mixed with a population of 500 000 responder cells. This result indicates that it is difficult to distinguish whether a small subpopulation of splenic adherent cells accounts for the CTL stimulation observed, or, alternatively, that nonadherent cells are able to stimulate primary CTL effector cells, although on a quantitative basis, less effectively than adherent cells. A further possibility is that a CTL-amplifying cell(s) requires Ia-positive SAC for induction. If so, the quantitative superiority of SAC and inferiority of G10-passed cells in the induction of CTL activity relative to unfractionated spleen cells might reflect the contribution of such amplifying cells. In this way, CTL precursors might be activated by alloantigen on all cell types, but the extent of clonal expansion, and hence the level of cytotoxicity observed, is influenced by the degree to which the CTL amplifier cell is also activated.

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