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STRAIN DIFFERENCES IN THE EXPRESSION OF THE EPA-1-RESTRICTING ELEMENT

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SUMMARY

Epa-1-specific cytotoxic T lymphocytes (CTL) lyse epidermal cells (EC) of different Epa-1$^+$ *H-2^k* strains, such as AKR, CBA, C58, and RF, at different levels. We used an H-2K^k-specific monoclonal antibody (mAb) to test the hypothesis that this phenomenon is due to differences in the H-2-restricting element. Initially, we established the specificity of this mAb for the Epa-1-restricting element by demonstrating its capacity to inhibit the lysis of CBA EC by Epa-1-specific CTL. We then used it as the probe in a cellular radioimmunoassay to quantify the expression of the restricting element by EC of different *H-2^k* strains. We found that C58 and RF EC bound significantly less of the mAb than did CBA EC. Although AKR also bound less of the mAb than did CBA EC, the difference was not statistically significant. To examine the generality of this phenomenon, we quantified the expression of K^k antigens on spleen cells (SC) of the same four strains. We found that RF SC, but not AKR or C58 SC, bound significantly less of the K^k mAb than did CBA SC. Thus, the differential CTL lysis of Epa-1$^+$ EC of different strains probably reflects differences in expression of the H-2-restricting element rather than of the nominal antigen.

INTRODUCTION

Epa-1 is a non-H-2 alloantigen that is expressed well on epidermal cells (EC), fibroblasts and activated macrophages but is poorly expressed, if at all, on lymphocytes (Steinmuller *et al.*, 1981a; Burlingham *et al.*, 1983, 1984). *In vivo*, Epa-1 functions as a minor histocompatibility antigen in the rejection of skin allografts and in local cutaneous graft-versus-host reactions (Steinmuller *et al.*, 1982; Tyler *et al.*, 1984). An antigen that cross-reacts with Epa-1 is expressed on EC of rats and humans (Steinmuller & Tyler, 1983). In genetic studies, *Epa-1* behaves as a single Mendelian locus (Steinmuller *et al.*, 1981b). The *Epa-1* gene does not appear to be very polymorphic: C3H/An, C3H/He, C3Heb/Fe, A/He, A/J, LG, and ST are Epa-1$^-$ but 43 other mouse strains are Epa-1$^+$ (Steinmuller *et al.*, 1985).

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Epa-1 is defined in mice by the specificity of *Ir* gene-regulated, IL-2-dependent, cytotoxic T lymphocytes (CTL) (Steinmuller *et al.*, 1981a; Tyler & Steinmuller 1981, 1982; Tyler *et al.*, 1982). Typically, Epa-1-specific CTL are raised in C3H/He hosts and are restricted by a gene product mapping to the *K* region of the *H-2* complex (Steinmuller *et al.*, 1981a; Tyler *et al.*, 1982). Recently Steinmuller and colleagues (1985) examined the susceptibility of EC of 25 different H-2K^k mouse strains to lysis by Epa-1-specific CTL; some of the results of their study are shown in Table 1. Note the broad range of values for EC targets from the four Epa-1$^+$ strains. Others have demonstrated a direct relationship between the level of expression of H-2-restricting elements and the susceptibility of cells to lysis by CTL directed against non-H-2 antigens (Flores & Gilmer, 1984; Flyer *et al.*, 1985; Meruelo, 1979; O'Neill & Blanden,

TABLE 1. Reactivity of Epa-1-specific cytotoxic T lymphocytes* with epidermal cells of five different H-2^k strains†

Target cells			
Strain	Epa-1 phenotype	No.‡	% specific lysis of epidermal cells mean ± SE
CBA/J	+	56	60·9 ± 1·6
AKR/J	+	19	46·5 ± 2·9
C58/J	+	4	44·5 ± 3·7
RF/J	+	15	32·5 ± 3·1
C3H/HeJ	−	58	5·8 ± 0·6

* Generated by priming C3H/He mice with CBA epidermal cells, then boosting host splenocytes by co-culturing them with irradiated CBA epidermal cells, as described in detail elsewhere (Steinmuller & Tyler, 1980).

† Data from Steinmuller *et al.*, 1985.

‡ Number of mice tested.

1979; Schmidt & Festenstein, 1982). Since Epa-1-specific CTL recognize Epa-1 only in the context of H-2K^k (Steinmuller *et al.*, 1981a), the differences in lysis of EC from different Epa-1$^+$ strains might reflect differences in the expression of either Epa-1 or H-2K^k antigens. The first alternative is not testable because, as for non-MHC antigens in general (Loveland & Simpson, 1986), Epa-1 does not appear to evoke a humoral response and such antibodies would be required to evaluate quantitative differences in Epa-1 expression on EC of different strains. However, the second alternative *is* testable because H-2K^k-specific antibodies are readily available. The purpose of the present investigation was to quantify possible differences in the expression of H-2K^k by EC of four representative Epa-1$^+$ strains, AKR, CBA, C58, and RF, that show different degrees of susceptibility to Epa-1-specific CTL.

MATERIALS AND METHODS

Mice

We purchased BALB/cByJ (*H-2^d*), C57BL/10 (B10) and C3H.SW (both *H-2^b*) and AKR/J, BIO.BR/SgSnJ, CBA/J, C3H/HeJ, C58/J and RF/J (all *H-2^k*) mice from the Jackson Laboratory, Bar Harbor, ME, and bred F_1 hybrid mice locally. B10.A(4R) mice (*H-2^{k}I^{k}D^b*)

were kindly provided by Dr John Niederhuber of the University of Michigan, Ann Arbor, MI. We used adult mice of both sexes as hosts in immunizations but selected the sex of the donor so as to preclude the induction of responses to the H-Y antigen (Wachtel, 1977).

Cell preparations and cell-mediated cytotoxicity (CMC) assays

We prepared mouse EC by trypsinization of tail and ear skin as described elsewhere (Steinmuller & Tyler, 1980). Briefly, we floated skin dermis-down on a solution of 0·5% (w/v) trypsin (Gibco, Grand Island, NY, U.S.A.; no. 610–5095) in phosphate-buffered saline (PBS) minus calcium and magnesium for 70–90 min at 37°C in a 5% CO_2 humidified incubator. We then separated the dermis and epidermis using fine forceps and rubbed the EC off the exposed surfaces with small glass probes into a solution of 0·025% (w/v) DNase (Sigma, St Louis, MO, U.S.A.; no. D-0876) and 10% (v/v) fetal calf serum (FCS) in Mishel–Dutton phosphate-buffered balanced salt solution (BSS). We filtered the resulting suspensions through 110 μ porosity nylon mesh (Tetko Inc., Elmsford, NY, U.S.A.) and centrifuged it at 300 ***g*** to pellet the EC. We used EC suspensions at this point as immunogens for *in vivo* and *in vitro* immunizations. We further purified EC and SC to be used as targets in the cellular radioimmunoassay (cRIA) by centrifugation over a Ficoll–Hypaque gradient (density 1·077) at 300 ***g*** for 15 min to remove non-viable and clumped cells. We filtered the EC used as targets in cell-mediated cytotoxicity (CMC) assays through a small amount of thoroughly washed glass-wool packed in a syringe barrel (Steinmuller & Tyler, 1980). We obtained mouse SC by teasing spleens in BSS containing 10% (v/v) FCS and filtering through the nylon mesh mentioned above. Our methods of generating Epa-1-specific CTL and assaying them in direct and cold-target inhibition 3-h chromium-release CMC assays are presented in detail elsewhere (Steinmuller & Tyler, 1980; Steinmuller *et al.*, 1981a; Tyler & Steinmuller, 1981).

Monoclonal antibodies (mAbs)

We obtained hybridomas 16-1-11N (ATCC no. HB-16) and 15-5-5S (ATCC no. HB-24) from The American Type Culture Collection, Rockville, MD, U.S.A. The antibodies secreted by these hybridomas are specific for H-2K^k and H-2D^k antigens, respectively (Ozato *et al.*, 1980). For the production of ascitic fluid, we passaged the hybridomas in histocompatible (C3H.SW × BALB/c)F_1 hybrid hosts and collected the fluid from the resulting ascites. We then centrifuged the ascitic fluid in a microfuge (Beckman, Fullerton, CA, U.S.A.; model B) for 1 min at 7,000 ***g*** to remove cells and debris. For the production of hybridoma culture supernatants, we grew the hybridomas to maximum cell density in culture and harvested the medium by passage through a filter with 0·2 μ porosity (Nalgene, Rochester, NY, U.S.A.; no. 120-0020). We aliquoted and stored the culture supernatant and ascitic fluid at −70°C until use. Immediately prior to use, we heat-inactivated aliquots of ascitic fluid by incubation in a 56°C water bath for 30 min.

CTL-blocking assays

We performed these assays as described by Epstein and co-workers (1980), with a slight modification. Briefly, we added 5 × 10^4 target cells in 20–100 μl aliquots of blocking antibody diluted in MEM containing 10% FCS and 25 mM HEPES buffer (MEM+) and incubated the trays for 30 min at 4°C. We then added effector cells to a volume of 100 μl. Otherwise, we performed these assays as described above for the CMC assays.

Cellular radioimmunoassay (cRIA)

We used a modification of a cRIA developed by Pierres and associates (1980). Briefly, we added 2·5 × 10^5 target cells to four 200-μl aliquots of antibody in MEM+ to wells of conical-bottomed microtitre trays. After incubation for 90 min at 4°C, we washed the wells three times by centrifugation for 8 min at 300 *g*, flicking out the supernatant, and resuspending the cells in 150 μl of PBS containing 0·2% (w/v) sodium azide and 0·2% (w/v) bovine serum albumin (BSA). We then added 1 × 10^5 counts per minute (cpm) of ^{125}I-labelled staphylococcal protein A (Amersham, Arlington Heights, IL, U.S.A.; no. IM.112) in 50 μl of PBS to each well. After incubation for 30 min at 4°C, we washed the wells four times and harvested their contents on to filter-paper discs using a cell harvester. We measured the radioactivity bound to the discs with a gamma counter (Beckman, Fullerton, CA, U.S.A.; Model 5500). We then calculated the mean of the replicates for each antibody sample after subtracting the average machine background cpm obtained by measuring the radioactivity registered by at least four blank counting tubes.

RESULTS

In order to evaluate whether or not EC of the AKR, C58, and RF mouse strains express reduced levels of the K^k-restricting element for Epa-1-specific CTL compared to CBA EC, we required a reagent that specifically recognizes the restricting element. For this purpose, we obtained hybridoma 16-1-11N which secretes a well-characterized mAb which binds to H-2K^k and does not cross-react with other *H-2*^{k} gene products (Ozato *et al.*, 1980; see Materials and Methods). However, the H-2K^k reactivity of this mAb does not guarantee its specificity for the antigen-restricting Epa-1-specific CTL because recent studies have shown that the *K* region of the *H-2* complex encodes at least two distinct class I antigens, though only

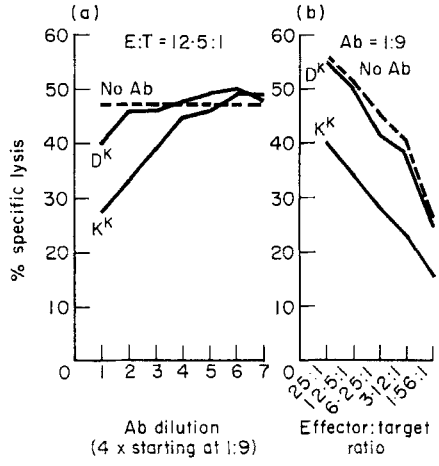

FIG. 1. Results of cytotoxic T-lymphocyte (CTL) blocking studies. Monoclonal antibodies (mAbs) with specificity for H-2K^k (K^k) and H-2D^k (D^k) antigens were assayed for the capacity to inhibit the lysis of Epa-1$^+$ CBA epidermal cells by Epa-1-specific CTL. (a) The effector to target cell ratio (E:T) was held constant at 12·5:1 and the antibody dilution was varied. (b) The antibody dilution was held constant at 1:9 and the E:T was varied. The broken line represents the level of target cell lysis in the absence of antibody.

one is expressed in all strains examined so far (Tryphonas *et al.*, 1983; Weiss *et al.*, 1984). Therefore, in the initial step of this project we tested the H-2K^k-specific mAb in CTL-blocking studies to establish its specificity for the Epa-1-restricting element and thus determine whether it could be used to quantify the expression of the element by EC of different mouse strains.

Figure 1a shows the results of an experiment in which the anti-H-2K^k mAb and an anti-H-2D^k control mAb were tested for their capacity to block the lysis of Epa-1$^+$ EC by Epa-1-specific CTL. Note the strong inhibition produced by the anti-K^k mAb compared to that by the anti-D^k mAb. We repeated this experiment on five separate occasions and obtained a similar degree of inhibition each time. In this particular experiment, the anti-D^k mAb caused some inhibition at the lowest antibody dilution, but this was not a consistent finding. Figure 1b shows that the anti-K^k antibody specifically inhibits target cell lysis over a broad range of effector to target (E : T) ratios. Thus, the anti-K^k mAb clearly reacts with the gene product that restricts Epa-1-specific CTL.

Previous studies have established the reliability of cRIAs in the quantification of molecules on the cell surface (Ada & Yap, 1979; Gerlier & Avice, 1984; O'Neill, 1984; O'Neill & Blanden, 1979). Therefore, we used the anti-K^k mAb as a probe in a cRIA to compare the expression of the Epa-1-restricting element by AKR, CBA, C58, and RF EC. We included H-2^d BALB/c EC to control for non-specific binding, and a sample composed of 80% CBA EC and 20% BALB/c EC as an internal standard; the latter cell mixture should

TABLE 2. Reactivity of H-2K^k-specific monoclonal antibody with epidermal cells (EC) of four H-2^k strains in the cellular RIA

Antibody dilution*	Radioactivity bound (cpm)†					
	CBA	RF	C58	AKR	80% CBA‡	BALB/c
1	1956 ± 94 (1943)	1452 ± 99 (1605)	1572 ± 48 (1448)	1756 ± 133 (1703)	1715 ± 139 (1649)	814 ± 78 (922)
2	1796 ± 87 (1775)	1274 ± 100 (1462)	1402 ± 33 (1292)	1650 ± 143 (1615)	1574 ± 102 (1514)	692 ± 47 (731)
3	1694 ± 95 (1671)	1202 ± 109 (1412)	1434 ± 37 (1311)	1555 ± 116 (1515)	1554 ± 85 (1488)	666 ± 57 (710)
4	1634 ± 91 (1612)	1148 ± 102 (1344)	1359 ± 32 (1243)	1494 ± 117 (1456)	1462 ± 93 (1400)	632 ± 50 (672)
5	1570 ± 103 (1547)	1120 ± 76 (1328)	1329 ± 48 (1207)	1422 ± 112 (1306)	1418 ± 111 (1352)	650 ± 43 (693)
6	1081 ± 84 (1065)	851 ± 51 (996)	996 ± 32 (911)	1051 ± 79 (1023)	1065 ± 85 (1019)	644 ± 45 (674)
7	844 ± 56 (828)	649 ± 23 (798)	829 ± 56 (741)	836 ± 87 (807)	842 ± 64 (795)	658 ± 56 (689)
Reagent control§	646 ± 52	494 ± 12	712 ± 49	662 ± 59	672 ± 44	599 ± 45

* Five-fold dilutions of 16-1-11N ascitic fluid starting at a dilution of 1 : 100.

† EC from the five strains were tested concurrently at the seven different antibody dilutions. Values are means ± SE of six experiments with adjusted means below in parentheses (see text).

‡ This sample was an internal standard composed of 80% CBA EC and 20% BALB/c EC.

§ Target cells were incubated with medium alone instead of with diluted antibody but otherwise were treated the same as the experimental groups.

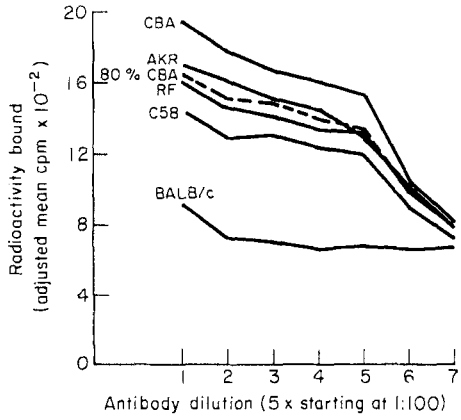

FIG. 2. Graphical presentation of the adjusted data from Table 2.

have expressed 20% fewer restricting molecules than 100% CBA EC. We tested all six EC samples concurrently at seven different antibody dilutions and repeated the assay six times.

The results from these experiments are presented in Table 2 in the form of both 'unadjusted' (raw) and 'adjusted' mean values. The rationale for adjusting the values is revealed by an inspection of the bottom row of the table. The number of counts bound in the absence of antibody (the reagent control) was different for EC of different strains. Clearly, this binding was non-specific and influenced the total binding observed in the presence of antibody. Thus, the purpose of adjusting the total binding values was to put the six strains on an equal footing with respect to this uncontrolled variable. To accomplish this, the reagent control was treated as the covariate in a randomized block analysis of covariance (Snedecor & Cochran, 1980) and adjusted means were obtained for total binding at the various antibody dilutions.

It is apparent from Fig. 2 that the mean binding values of AKR, RF, and C58 EC were lower than those of CBA EC at all antibody dilutions. In fact, an analysis of covariance revealed significant differences ($P < 0{\cdot}001$) among the strains at each dilution. Pair-wise comparisons of these data using Dunnett's procedure (Dunnett, 1955) revealed that the

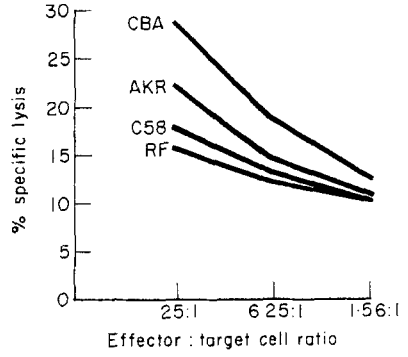

FIG. 3. Reactivity of H-2K^k-specific cytotoxic T lymphocytes with EC of strains CBA, AKR, C58, and RF (all H-2K^k). Effector cells were raised in C57BL/10 (*H-2*^{b}) hosts immunized with spleen cells from the *H-2* congenic strain B10.A(4R) (*H-2K*^{k}*I*^{k}*D*^{b}). Values are means of seven assays.

TABLE 3. Reactivity of Epa-1-specific cytotoxic T lymphocytes generated by immunizing C3H/He mice with RF or C58 epidermal cells

Immunizing cell strain	Target cell strain	Mean % specific lysis ±SE at E:T = 25:1 (four tests)
RF	C3H/He	1·1 ± 1·1
	RF	28·5 ± 4·2
	AKR	43·0 ± 2·9
	CBA	54·9 ± 3·1*
C58	C58	49·3 ± 8·0
	CBA	66·0 ± 5·9†

* $P < 0{\cdot}01$ *vs.* RF.
† $0{\cdot}05 > P > 0{\cdot}25$ *vs.* C58.

values for C58 and RF EC were significantly different ($P < 0{\cdot}05$) from the values for CBA EC at dilutions 1–5. Thus, C58 and RF EC clearly express fewer K^k-restricting molecules than do CBA EC. Whether AKR EC actually express fewer restricting molecules than CBA EC is less clear because the difference between their mean values was significant only at dilution 5.

As an additional approach to evaluate the relative level of K^k expression by EC of the four strains, we assayed their susceptibility to lysis by H-2K^k-specific CTL. As seen in Fig. 3, the mean per cent specific lysis values of AKR, C58 and RF EC were lower than those of CBA EC at all E:T ratios. An analysis of variance of these data showed that the differences between RF and CBA, and between C58 and CBA, were statistically significant ($P < 0{\cdot}05$) at the highest E:T ratio. Thus, these data corroborate the findings obtained using the cRIA.

Previously, we found that CTL populations generated by immunizing C3H/He mice with EC of any of several other H-2^k strains are directed predominantly against Epa-1, with only minor reactions against other non-H-2 antigens (Steinmuller *et al.*, 1981a; Tyler and Steinmuller, 1981). However, to test for possible quantitative differences among Epa-1 antigens of different Epa-1^+ strains, we immunized C3H/He hosts with C58 or RF EC, the EC least susceptible to lysis by Epa-1-specific CTL, and assayed the CTL so generated with those EC targets, as well as with CBA EC, most susceptible to Epa-1-specific CTL. Indeed, the CTL generated with C58 and RF immunogens still lysed CBA EC targets at higher levels (Table 3). Moreover, in cold-target inhibition assays, CBA EC always were the best inhibitors of Epa-1-specific CTL, and RF always the worst (data not shown).

Finally, to determine whether the strain differences in K^k expression by EC also held for lymphoid cells, we assayed SC from the same five strains exactly as described above for the EC quantification experiments with the exception that SC were substituted as targets. The average results of six such tests are presented in Fig. 4. These data were also subjected to a randomized block analysis of covariance. In this case, the analysis showed that the reagent control values were not significantly related to total binding in the presence of antibody. Hence, the reagent control values were excluded from the analysis and a randomized block analysis of variance was run at each antibody dilution. Figure 4 shows that the mean binding values of RF SC were clearly lower than those of the other three strains at each dilution. Pairwise comparisons of these data using Dunnett's procedure confirmed that the values for RF

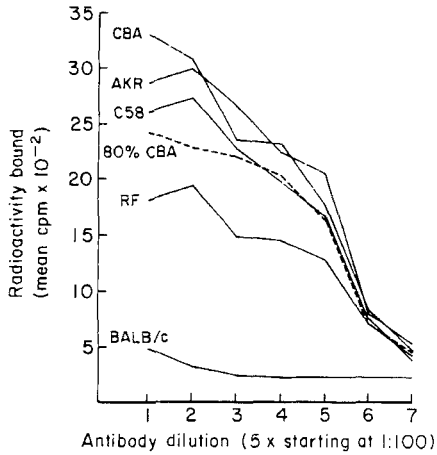

FIG. 4. Reactivity of H-2K^k-specific monoclonal antibody with spleen cells (SC) of four *H-2*^{k} strains in the cellular RIA. SC from the five strains and an internal standard (a mixture of 80% CBA SC and 20% BALB/c SC) were tested concurrently at the seven different antibody dilutions. Values are means of six assays.

SC were significantly lower ($P < 0{\cdot}05$) than those of CBA SC at dilutions 1–5. Conversely, the values for AKR and C58 SC were not significantly lower ($P < 0{\cdot}05$) than those of the CBA SC at any of the dilutions.

DISCUSSION

T cells recognize foreign antigen only in the context of self molecules encoded within the MHC—a phenomenon referred to as MHC restriction (Katz *et al.*, 1973; Rosenthal & Shevach, 1973; Zinkernagel & Doherty, 1979). We have demonstrated the capacity of H-2K^k-reactive antibodies to block the lysis of Epa-1$^+$ EC by Epa-1-specific CTL. These results are consistent with the well known capacity of H-2-reactive antibodies to block the lysis of target cells by CTL directed against non-H-2 alloantigens (Fischer Lindahl & Lemke, 1979; Koo *et al.*, 1979). Our findings are also consistent with those of Steinmuller and colleagues (1981a) who had previously mapped the restricting element for Epa-1-specific CTL to the *K* region of the *H-2* complex. We found that the H-2K^k-specific mAb 16-1-11N caused a marked inhibition of target cell lysis over a broad range of E : T ratios. This inhibition was specific because the H-2D^k-specific mAb 15-5-5S, which also binds to the target cells, failed to cause inhibition except at the highest antibody concentration. Thus, it seems clear that 16-1-11N does, in fact, recognize the H-2-restricting element for Epa-1-specific CTL.

At the highest antibody concentration used, 16-1-11N inhibited target cell lysis to 40% of that of the negative control value. We had expected to observe somewhat greater inhibition because Epstein and colleagues (1980) had reported that 16-1-11N completely blocks the lysis of target cells by H-2K^k-specific CTL. However, the Epa-1-specific CTL used in our studies were raised in C3H/He mice and, as such, also expressed H-2K^k antigens. Thus, competitive absorption by K^k antigens present on the effector cells may have accounted for the less than complete inhibition by 16-1-11N in our studies. Indeed, Epstein and colleagues (1980) have shown that competitive absorption of an antibody by effector cells can completely eliminate its capacity to inhibit target cell lysis by CTL. The aforementioned inability of the H-2D^k-specific control mAb to inhibit target cell lysis indicates that the binding of antibody to the effector cells *per se* has little effect on their lytic activity.

We utilized mAb 16-1-11N as the probe in a cRIA to demonstrate differential levels of H-$2K^k$ expression by EC of different *H-2*^{k} strains. These differences correlated closely with the susceptibility of the strains to lysis by Epa-1-specific CTL. Thus, the simplest explanation for the differential susceptibility of EC of different strains to lysis by Epa-1-specific CTL lies in the differential expression of the H-2-restricting element. These findings are consistent with the results of earlier studies that have shown that recognition of the H-2-restricting element—rather than recognition of the target antigen itself—is the major limiting factor in the lysis of target cells by H-2-restricted CTL (Flores & Gilmer, 1984; Flyer *et al.*, 1985; Meruelo, 1979; O'Neill & Blanden, 1979; Schmidt & Festenstein, 1982).

Our findings do not rule out the possibility that differences in *Epa-1* gene expression might also influence the susceptibility of the strains to lysis by Epa-1-specific CTL. In principle, such differences in *Epa-1* gene expression could be quantitative or qualitative. Unfortunately, it is probably impossible to test for quantitative differences in Epa-1 gene expression without anti-Epa-1 antibody. However, if *qualitative* differences at the *Epa-1* locus do exist between mouse strains, they are, at best, extremely limited because, regardless of the strain origin of the EC immunogens against which they are raised, Epa-1-specific CTL consistently show the same strain reactivity pattern: CBA > AKR > C58 > RF (Table 3 and Steinmuller *et al.*, 1981a). Thus the same Epa-1 antigens are apparently expressed by EC of these strains. However, the interpretation of these data is complicated by the fact that CBA mice express higher levels of the H-2-restricting element than do EC of the other strains. Hence, the dominating influence of this factor on the lytic activity of Epa-1-specific CTL would have obscured the detection of any subtle Epa-1 polymorphisms.

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