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INDUCTIVE REQUIREMENTS FOR THE GENERATION OF VIRUS-SPECIFIC T LYMPHOCYTES

III. Production of Target Cells Lysable by Poxvirus-Specific and Allospecific Cytotoxic T Lymphocytes with Membrane Fragments Bearing Viral and H-2 Antigens[1]

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Susceptibility to *in vitro* lysis by H-2 restricted virus-specific and/or allospecific cytotoxic T lymphocytes (CTL) was transferred to normally insusceptible fibroblastic cells by exposing them to membrane fragments of donor cells expressing vaccinia virus (VAC) antigens together with different alloantigens. After the simultaneous transfer of both types of antigens, the recipient "membrane hybrid" targets were highly lysable by alloreactive CTL specific for H-2 antigens of the donor cells as well as by VAC-specific CTL having the same H-2 haplotype as recipient cells. However, these same targets were lysed only minimally by VAC-specific CTL of donor cell haplotype.

When used to prime H-2 identical mice, membrane fragments induced only VAC-specific CTL. Priming of H-2 nonidentical mice induced CTL of two major specificities, as descriminated by cold target competition; one for H-2 antigens of the cells used to prepare membrane fragments, and the other for VAC. Lysis by the latter was always restricted to viral antigen-bearing targets that shared H-2 identity with the primed mice. The same outcome was obtained when membrane fragments were used to stimulate primary CTL induction *in vitro*, in which case fragment presentation by adherent cells was required.

Collectively, the results of this study indicate that viral and H-2 determinants that comprise the "virus + self" complex can dissociate, retaining their respective native identities within target or stimulator cell membranes.

The specificity of murine cytotoxic T lymphocytes (CTL)[3] is directed toward antigenic determinants coded for by genes mapping mainly to the K/D regions of the major histocompatibility (H-2) complex (1, 2). Broadly speaking, there are two subclasses of CTL that recognize and lyse target cells expressing, respectively, non-self H-2 antigens (alloantigens) (3) and other types of antigens in association with self H-2 K/D determinants. In the latter case, H-2-restricted CTL with specificities for minor histocompatibility antigens (4), haptens (5), the H-Y antigen and viral antigens (6–8) have been demonstrated, and they appear to be less cross-reactive than their allospecific counterparts (9–11).

The requirement for joint recognition of antigen and H-2 antigens by H-2-restricted CTL appears to be imposed during intrathymic maturation of their precursors (12), whereas the role of the thymus in generating the repertoire of alloreactive CTL remains unclear (13). Primary (1°) induction of both CTL subclasses is dependent on the participation of antigen-presenting cells, presumed to be macrophages (14, 15). Products of the H-2I subregions of these cells also appear to be important during induction of 1° (16–18) but not secondary (2°) (8, 19–21) H-2-restricted CTL responses. Although H-2 determinants must be expressed by target cells in order for them to be lysed by either alloreactive or H-2 restricted CTL, it remains to be determined whether there are fundamental differences in the way these determinants are recognized by each CTL subclass.

In the previous accompanying paper (19) we showed that the immunogenic integrity of "virus + self" complexes, formed by the interaction of vaccinia virus (VAC) with murine fibroblastic cells, was preserved in purified cell membrane fragments. In the present study, such membrane fragments were used to transfer these complexes to H-2 disparate recipient cells. Fibroblastic recipients were assessed for their relative susceptibility to lysis by alloreactive and VAC-specific CTL whose specificities were dictated by the H-2 haplotype of either the fragment donors or recipients. Macrophage recipients were used to stimulate 1° induction of both CTL subclasses *in vitro*. Under these conditions, we were able to obtain evidence that viral and H-2 antigens comprising the transferred complexes are dissociable and are recognized as independent entities during both the inductive and effector phases of CTL responses.

MATERIALS AND METHODS

Virus. The same preparations of infectious and ultraviolet (UV) irradiated VAC (WR strain) described in the accompanying paper (19) were used in the present study. For some

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[3] Abbreviations used in this paper: CTL, cytotoxic T lymphocyte(s); HBSS, Hanks' balanced salt solution; 1°, primary; 2°, secondary; VAC, vaccinia virus.

experiments, similarly prepared VAC of the same strain was kindly donated by Dr. B. Moss, National Institute of Allergy and Infectious Diseases.

Animals. Female C3H/He (H-2^k) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Preparation of target, stimulator and competitor cells. The methods used are treated briefly here. More details are given in the accompanying paper (19) and in previous reports (8, 22). Usually, target, stimulator, and competitor cells were prepared in essentially the same fashion. In each case, L (C3H, H-2^k), 3T3 (BALB/c, H-2^d), and for some experiments, MC57G (C57BL/6, H-2^b) cells suspended in HBSS were exposed to UV-inactivated VAC for 10 min at 4°C and then washed to remove unadsorbed virus. When used as targets, cells were labeled with ^{51}Cr (New England Nuclear, Boston, Mass.) before exposure to virus. For certain experiments, indicated in the text, target cells were prepared by using infectious VAC at a multiplicity of 20 plaque-forming units (PFU) per cell. Target, stimulator, or competitor cells exposed to UV-inactivated virus are subsequently referred to as L-VAC, 3T3-VAC, and MC57G-VAC. When placed in culture with lymphocytes, the cells were suspended in a standard medium of RPMI 1640 containing 10% fetal calf serum (FCS; Grand Island Biological Co., Grand Island, N. Y.), 5×10^{-5} M 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (40 μg/ml).

Preparation of intact and fragmented cell membranes. The method employed was essentially that of Warren and Glick (23) and is described in detail in the previous paper (19). Membrane fragments used to transfer H-2 and viral antigens were added to suspensions of recipient target or stimulator cells (10^7 cells/ml) in standard medium without FCS at a ratio of 1 cell-equivalent per recipient cell. (A cell-equivalent refers to fragments obtained from one intact plasma membrane.) The mixtures were incubated for 1 hr at 37°C and then washed twice to remove free fragments.

Immunization. All mice were immunized i.v. at 8 to 10 weeks of age. For virus priming, a single dose of either 2×10^4 PFU of infectious VAC or 3×10^7 particles of UV-inactivated VAC was given. When priming with membrane fragments, each mouse was inoculated with 2×10^6 cell-equivalents in Hanks' balanced salt solution (HBSS).

Induction of 1° and 2° CTL in vitro. Spleen cells free of erythrocytes were prepared as previously described (19) from normal 6- to 8-week-old mice and suspended in standard medium at a concentration of 10^6 viable cells/ml. When used for induction of 1° CTL with membrane fragments, 25-ml aliquots together with 25×10^6 cell-equivalents of various preparations of fragments were cultured for 5 days in 25 cm^2 plastic tissue culture flasks (Costar, Cambridge, Mass.) at 37°C in air containing 5% CO_2. Alternatively, membrane fragments were first added to splenic macrophages, which in turn were used to "present" the fragments either to unfractionated responder spleen cells or to those depleted of adherent cells by double passage through nylon wool columns (24). Macrophages (adherent cells) were prepared by culturing spleen cells at a density of $5 \times 10^6/cm^2$ in plastic dishes 60 mm in diameter (Costar) for 4 hr at 37°C followed by removal of nonadherent cells by repetitive washing with HBSS. Macrophages were detached with a rubber policeman, suspended in standard medium, and aliquots containing 2.5×10^6 cells were mixed with an equal number of membrane fragment cell-equivalents in a total volume of 0.5 ml. The mixtures were incubated for 1 hr at 37°C, after which free fragments were removed by washing with HBSS, and then each aliquot was added to a corresponding flask of responder cells. Control flasks contained nylon wool column-passaged responders to which membrane fragments were added directly. Alloreactive 1° CTL were induced in one-way mixed lymphocyte cultures (MLC) as described in the previous paper (19).

To induce VAC-specific and allospecific 2° CTL, spleen cells from membrane fragment-primed mice and control normal mice were cultured for 5 days at 37°C with L, L-VAC, 3T3, and 3T3-VAC stimulators in standard medium, as described previously (8, 19).

Assays of CTL. All cytotoxicity assays were performed with both H-2 identical and nonidentical target cells as described previously (19). Targets of each haplotype, consisting of cells exposed to virus, cells exposed to membrane fragments, or normal cells, were seeded into flat-bottomed wells of Linbro microtiter plates (Linbro Chemical Co., New Haven, Conn.) in 0.1-ml aliquots containing 2×10^4 cells. Spleen cells to be assayed for 1° or 2° CTL, as well as appropriate control spleen cells, were added at varying effector:target ratios in triplicate or quadruplicate 0.1 ml volumes, and the mixtures were incubated for 6 hr at 37°C in air containing 5% CO_2. At the end of this period, 0.1 ml of culture medium was transferred from wells to corresponding 12 x 75 mm glass tubes, which were counted in a Packard autogamma spectrometer (Packard Instrument Co., Downers Grove, Ill.).

The percentages of virus-specific or allospecific lysis ± the standard error were calculated by using data from assays in which target cells, cultured in the absence of lymphocytes, spontaneously released 15% or less of their label and in which the standard error was ≦3%. For the sake of clarity, lysis of 3% or less is indicated as zero in the accompanying tables.

RESULTS

Target cell formation by antigen transfer with membrane fragments. The membrane fragments used in this study generally took the form of small vesicles having an average diameter of 200 nm, as viewed by electron microscopy (A. J. Hapel, unpublished observation). Membrane vesicles have a propensity to fuse with the plasma membranes of any intact cells that they encounter and with one another (25, 26). The fusing efficiency of vesicles depends to some extent on their lipid composition and charge (27, 28), but fusion occurs in a relatively nonspecific manner (29, 30) and can be enhanced by the presence of certain viral antigens (31).

As shown in Table I, L-VAC and 3T3-VAC cell membrane fragments were very effective vehicles for the transfer of H-2 antigens. Normally insusceptible fragment-recipient cells became highly lysable by MLC-generated CTL with specificity for H-2 determinants of fragment-donor cells. For example, recipient L (H-2^k) and MC57G (H-2^b) cells, after exposure to donor 3T3-VAC (H-2^d) cell-membrane fragments, were lysed as effectively as 3T3 targets by anti-H-2^d CTL. Recipients of H-2 antigens transferred with fragments of normal L or 3T3 cells exhibited the same patterns of susceptibility to lysis by allospecific CTL (data not shown).

Recipients of L, L-VAC, 3T3, and 3T3-VAC fragments were also tested for susceptibility to lysis by H-2-restricted VAC-specific CTL. The results are illustrated by the following example taken from Table II. Either L-VAC or 3T3-VAC fragments readily transferred viral antigen to both L and 3T3 cells, causing them to become lysable by, respectively, C3H anti-VAC

TABLE I

Acquired susceptibility to allospecific lysis by transfer of H-2 antigens with membrane fragments

CTL Specificity[a]	Fragment Donor[b]	Target Recipient[c]	Specific Lysis at E/T Ratios of[d]		
			20	10	5
			%		
Anti-H-2^d		3T3(H-2^d)	49.1	34.9	29.0
(C3H anti-BALB/c)	3T3-VAC	3T3	61.6	51.8	39.6
		L(H-2^k)	0	0	0
	3T3-VAC	L	55.3	49.7	36.9
		MC57G(H-2^b)	4.6	0	0
	3T3-VAC	MC57G	56.2	45.0	30.7
Anti-H-2^k		L	76.1	58.3	48.5
(BALB/c anti-C3H)	L-VAC	L	79.1	59.7	47.8
		3T3	0	0	0
	L-VAC	3T3	68.8	55.7	39.9
		MC57G	6.5	5.0	3.8
	L-VAC	MC57G	78.2	69.6	56.7

[a] CTL were generated in one-way mixed lymphocyte cultures.

[b] Fragments of purified plasma membranes of L and 3T3 cells which had been exposed to UV-irradiated VAC particles (500/cell) for 10 min at 4°C.

[c] Cells labeled with ^{51}Cr and exposed, where indicated, to UV-irradiated VAC particles (300/cell) for 10 min at 4°C and/or to an equal number of membrane fragment cell-equivalents for 1 hr at 37°C.

[d] Effector/target ratio. Specific lysis of each target in these and all subsequent CTL assays was determined from the amounts of radioactive label (cpm) released in the presence of lymphocytes minus that released spontaneously from targets alone during a 6 hr period. Calculations were based on fractions of released cpm by using input cpm as a constant denominator. Label released spontaneously usually ranged from 5 to 10% of input and never exceeded 15%.

TABLE II

Acquired susceptibility to H-2 restricted VAC-specific lysis by transfer of viral and/or H-2 antigens with membrane fragments

CTL Specificity[a]	Fragment Donor[b]	Target Recipient[c]	Specific Lysis at E/T Ratios of		
			40	20	10
			%		
C3H (H-2^k)		L-VAC	68.4	59.7	44.4
anti-VAC		L	4.7	0	0
	L-VAC	L	58.6	47.5	39.2
	3T3-VAC	L	38.9	31.7	22.6
		3T3-VAC	0	0	0
	L	3T3-VAC	24.4	14.3	9.1
		3T3	0	0	0
	L-VAC	3T3	15.4	11.2	8.8
BALB/c (H-2^d)		3T3-VAC	48.1	36.7	28.9
anti-VAC		3T3	0	0	0
	3T3-VAC	3T3	39.8	28.5	25.5
	L-VAC	3T3	42.6	33.9	24.5
		L-VAC	0	0	0
	3T3	L-VAC	31.0	19.4	8.1
		L	0	0	0
	3T3-VAC	L	14.7	11.5	8.4

[a] CTL from spleens of mice primed 6 days previously with 3×10^7 particles of UV-irradiated VAC.

[b] Fragments prepared as described for Table I.

[c] Targets prepared as described for Table I except that 3T3-VAC recipients of L fragments, and L-VAC recipients of 3T3 fragments were exposed to infectious VAC (20 PFU/cell) for 10 min at 4°C.

CTL and BALB/c anti-VAC CTL. (Compare with lysis of L-VAC and L targets by the former, and lysis of 3T3-VAC and 3T3 targets by the latter.) However, lysis by VAC-specific CTL was much less impressive when the fragment-recipient target cells differed from the effector cells at H-2. Thus, 3T3 recipients of L-VAC fragments and L recipients of 3T3-VAC fragments were lysed poorly by anti-VAC CTL from, respectively, C3H and BALB/c mice. The significance of this finding will be dealt with later (see *Discussion*), but it should be kept in mind that these same fragment recipients were lysed very effectively by alloreactive CTL specific for H-2 antigens of the fragment donors (Table I) and therefore were expressing both viral and H-2 antigens.

Alloantigen transfer was also effective in overriding the H-2-restricted susceptibility of recipient target cells to lysis by VAC-specific CTL. As shown in Table II, 3T3-VAC and L-VAC became lysable by, respectively, C3H anti-VAC and BALB/c anti-VAC CTL after exposure to fragments of normal cells sharing H-2 K/D antigens with the CTL. For reasons to be discussed subsequently, these results were obtained only when the recipient targets were prepared by using infectious virus.

In vivo priming with membrane fragments. The results described above suggested that although VAC and H-2 were recognized jointly by virus-specific CTL, their association was not irreversible. When expressed on membrane fragments, they seemed free to associate with viral or H-2 determinants of recipient target cells, enabling the latter to be lysed by H-2 nonidentical CTL. However, these results did not exclude the possibility that membrane fragments also expressed complexes in which the antigenic integrity of VAC and H-2 was subverted in a manner favoring the formation of a stable "altered self" (32–34). If this were the case, then mice primed with VAC-bearing membrane fragments prepared from H-2 nonidentical cells might be expected to generate CTL specific for virus + alloantigen in addition to the alloantigen alone and virus + self.

C3H and BALB/c mice each were inoculated with 2×10^6 cell-equivalents of membrane fragments prepared from either L-VAC, 3T3-VAC, MC57G-VAC, or the corresponding normal cells. Six days later, their spleens were assayed for 1° CTL activity against L, L-VAC, 3T3, and 3T3-VAC targets. The results (Table III) indicated that, in mice of each strain, CTL

TABLE III

Specificities of 1° CTL induced in vivo by priming with membrane fragments bearing both VAC and H-2 antigens

Responder	Membrane Fragments Used to Prime[a]	E/T	Targets Specifically Lysed by 1° CTL			
			L-VAC	L	3T3-VAC	3T3
					%	
C3H(H-2^k)	L-VAC (H-2^k)	40	35.6	8.6	0	0
		20	28.4	6.9	0	0
		10	23.2	0	0	0
	3T3-VAC (H-2^d)	40	27.7	8.2	24.2	28.6
		20	20.0	3.4	17.4	20.1
		10	14.7	0	10.2	10.1
	MC57G-VAC (H-2^b)	40	29.8		0	0
		20	21.4		0	0
		10	10.9		0	0
BALB/c(H-2^d)	L-VAC	40	32.0	36.7	25.6	0
		20	24.1	27.9	19.7	0
		10	19.6	20.2	11.9	0
	3T3-VAC	40	0	7.5	28.7	0
		20	0	5.4	20.3	0
		10	0	0	14.1	0
	MC57G-VAC	40	0		21.6	0
		20	0		11.7	0
		10	0		8.9	0

[a] Mice were immunized i.v. with 2×10^6 cell-equivalents of each preparation of membrane fragments and their spleens were assayed for CTL 6 days later.

of only two specificities were induced, one for H-2 native to the membrane fragments, and the other for virus + self (VAC together with H-2 antigens of the immunized animals). Allospecific lysis of both normal targets (L or 3T3) and those expressing VAC did not differ significantly. Although not shown, priming with membrane fragments of normal cells induced only alloreactive CTL whose lytic activity was not significantly different from that of CTL induced by corresponding L-VAC or 3T3-VAC fragments.

In vitro restimulation of membrane-primed T cells. We have shown previously that VAC-specific 2° CTL can be induced very effectively by culturing L-VAC or 3T3-VAC stimulators with H-2 identical (but not nonidentical) virus-primed spleen cells that have been enriched for T lymphocytes and macrophage depleted by double passage through nylon wool columns (8). Antigen presentation is accomplished directly by the stimulator cells, thereby eliminating the possibility of viral antigens being transferred to and presented by "self" macrophages in the responder population. This procedure was used in the present study for determining the specificities of 2° CTL responses by T cells from C3H and BALB/c mice primed 40 days previously with membrane fragments of either L-VAC, 3T3-VAC or the corresponding normal cells. The patterns of responses obtained (Table IV) were very similar to those induced *in vivo* (Table III), being specific for either VAC + self or alloantigens. As a representative example, BALB/c mice developed H-2^d-restricted VAC-specific 1° CTL after being primed with either 3T3-VAC or L-VAC fragments (Table III). However, *in vitro*, potent 2° CTL of the same specificity could be obtained only by restimulating their T cells with 3T3-VAC. On the other hand, BALB/c T cells, previously primed and restimulated with L-VAC, yielded only anti-H-2^k CTL. Allospecific 2° CTL responses induced by stimulation with control L and 3T3 cells were essentially identical to those induced by L-VAC and 3T3-VAC (data not shown). Thus, the results in Table IV provided further evidence that viral and H-2 determinants simultaneously expressed on stimulator/target cell membranes are recognized as separate entities by T cells.

Discrimination between virus-specific and allospecific CTL by cold target competition. These experiments were done to further exclude the possibility that priming and restimulation with L-VAC or 3T3-VAC fragments might have induced CTL specific for virus + alloantigen. Mixtures of cold (unlabeled) and labeled targets (16:1, respectively) were cultured overnight with 2° CTL. The latter were induced as described above and characterized, by their lytic activity, as either allospecific or virus-specific, as shown in Table IV. Lysis in the presence of competitors was compared with that in corresponding cultures containing CTL and labeled targets only. The patterns of inhibition shown in Table V indicated that 2° CTL induced by stimulator cells expressing "virus + alloantigen" were specific for the alloantigen alone. Thus, 92% of the lytic activity exhibited by BALB/c CTL against L-VAC targets was inhibited in the presence of L competitors, and 81% of C3H anti-3T3-VAC activity was inhibited in the presence of 3T3 competitors.

In vitro 1° CTL induction with membrane fragments. Membrane fragments were used to induce 1° CTL responses *in vitro* in order to determine: 1) whether the specificities of such responses were the same as those obtained by *in vivo* priming, and 2) whether CTL induction required fragment presentation by adherent cells (macrophages). Fragments prepared from L-VAC and 3T3-VAC cells were cultured with normal responder spleen cells from either C3H or BALB/c mice. To responders that were either unfractionated or macrophage depleted, fragments were added either alone or in association with macrophages, as described in *Materials and Methods.* After 5 days incubation at 37°C in air containing 5% CO_2, the cultures were tested for lytic activity against L-VAC and L target cells. The data presented in Table VI are representative of three experiments, and they confirm two points made previously from the results of *in vivo* priming (Table III). First, C3H spleen cells responded to both L-VAC (H-2^k) and 3T3-VAC (H-2^d) fragments by producing virus-specific CTL whose lytic activity was restricted to H-2^k targets. Second, BALB/c spleen cells responded to L-VAC fragments by producing CTL with equal activity against both L-VAC and L targets. This activity in other experiments (not shown) was inhibited almost completely by cold L competitors. In addition, induction of both VAC-

TABLE IV

Specificities of 2° CTL induced by in vitro restimulation of VAC and H-2 antigen-bearing membrane fragment-primed T cells

Responder[a]	Membrane Fragments Used to Prime	Stimulator Cells[b]	E/T	Targets Specifically Lysed by 2° CTL[c]			
				L-VAC	L	3T3-VAC	3T3
				%			
C3H(H-2^k)	L-VAC (H-2^k)	L-VAC	10	*76.3*	24.1	0	0
			5	*71.9*	12.2	0	0
		3T3-VAC	10	22.5	6.8	0	0
			5	17.6	5.1	0	0
	3T3-VAC (H-2^d)	L-VAC	10	*69.5*	11.1	8.6	7.6
			5	*58.7*	0	7.9	5.1
		3T3-VAC	10	24.4	8.6	(51.2)	(49.6)
			5	17.6	0	(40.6)	(35.7)
BALB/c(H-2^d)	L-VAC	L-VAC	10	(63.9)	(72.6)	0	0
			5	(51.4)	(59.7)	0	0
		3T3-VAC	10	12.4	21.2	*53.1*	0
			5	9.5	17.7	*42.6*	0
	3T3-VAC	L-VAC	10	18.3	12.7	12.6	0
			5	12.1	6.1	9.7	0
		3T3-VAC	10	12.4	13.1	*65.4*	3.7
			5	7.3	8.4	*52.7*	0

[a] Nylon wool column-passaged spleen cells from mice primed 40 days previously with membrane fragments as described for Table III.

[b] L and 3T3 cells exposed to UV-irradiated VAC particles (300/cell) for 10 min at 4°C.

[c] Percentages of virus-specific lysis are italicized; percentages of allospecific lysis are in parentheses.

TABLE V

Discrimination between anti-viral and anti-H-2 CTL specificities by cold target competition

Responder[a]	Membrane Fragments Used to Prime	Stimulator Cells	Labeled Target	Cold Target	Specific Lysis at E/T Ratio of 10 with Cold Targets[b]	
					Absent	Present
					%	
BALB/c	3T3-VAC	3T3-VAC	3T3-VAC	L	65.4	58.2 (11.0)
				3T3-VAC	65.4	11.8 (82.0)
	L-VAC	L-VAC	L-VAC	L	63.9	5.1 (92.0)
				3T3-VAC	63.9	58.7 (8.1)
C3H	3T3-VAC	3T3-VAC	3T3-VAC	3T3	51.2	9.7 (81.1)
				L-VAC	51.2	38.0 (25.8)
	L-VAC	L-VAC	L-VAC	3T3	76.3	67.1 (12.1)
				L-VAC	76.3	12.9 (83.1)

[a] Spleen cells from mice primed with membrane fragments (as described for Table III) were restimulated *in vitro*. Lysis by 2° CTL was assayed in the presence or absence of cold targets. The ratio of cold/labeled targets was 16.

[b] Figures in parentheses are the percentages of inhibition by cold targets.

TABLE VI

In vitro induction of virus-specific and alloreactive 1° CTL with VAC and H-2 antigen-bearing membrane fragments

Stimulator Membrane Fragments ± Adherent Cells	Responding Spleen Cells[a]		Targets Specifically Lysed by 1° CTL					
			L-VAC			L		
	Source	Treatment	E/T 40	20	10	40	20	10
			%					
L-VAC (−)	BALB/c	None	35.2	26.7	17.9	34.7	27.9	21.2
	C3H		41.6	32.7	21.4	8.6	7.1	6.7
L-VAC (+)	BALB/c	None	67.8	58.4	49.6	54.9	48.7	38.1
	C3H		63.4	51.2	38.9	11.3	11.9	7.8
L-VAC (−)	BALB/c	Depleted of adherent	0	0	0	0	0	0
	C3H	cells	0	0	0	14.5	11.2	10.8
3T3-VAC (−)	BALB/c	None	4.8	0	0	7.6	5.1	4.8
	C3H		27.6	17.5	9.2	6.4	0	0
3T3-VAC (+)	BALB/c	None	3.8	0	0	6.1	5.8	0
	C3H		32.4	24.8	14.6	4.6	0	0
3T3-VAC (−)	BALB/c	Depleted of adherent	7.6	3.7	0	9.2	8.4	7.3
	C3H	cells	0	0	0	5.9	7.3	0

[a] Spleen cells, either unfractionated or depleted of adherent cells by nylon wool column-passage, were cultured with equal numbers of membrane fragment cell-equivalents for 5 days. Fragments were added to responders with (+) or without (−) syngeneic adherent cells.

specific and allospecific CTL with membrane fragments required the participation of macrophages, and was enhanced if the macrophages were preincubated with fragments.

DISCUSSION

The primary aim of this study was to determine whether targets or stimulators of VAC-specific CTL express viral and H-2 antigens in the form of stable complexes that possess unique determinants (altered-self) or whether these antigens randomly associate with one another while maintaining their respective identities. In order to discriminate between these two possibilities, we prepared purified plasma membrane fragments from normal fibroblastic cells and from cells previously exposed to UV-irradiated VAC. These fragments were used in experiments of two general types; 1) transferring of viral and/or H-2 antigens to normal or VAC-bearing H-2 nonidentical cells, which were employed *in vitro* as targets of both VAC-specific and allospecific CTL, and 2) immunizing both H-2 identical and nonidentical mice whose spleens were assayed for 1° VAC-specific and allospecific CTL.

In designing experiments of the first type, we reasoned that if viral antigens expressed by fragment donor cells were irreversibly complexed with H-2 antigens, transfer of such complexes to H-2 nonidentical cells should render the latter more susceptible to lysis by VAC-specific CTL of fragment-donor H-2 haplotype than by CTL of recipient haplotype. On the other hand, a converse pattern of lysis would indicate that the transferred viral antigens were free to associate with H-2 antigens of the recipient cells. Implicit in this line of reasoning were two assumptions. The first, founded on previous data (19), was that no viral antigens were transferred by free (nonfused) VAC particles. The second assumption was that if viral and H-2 antigens were transferred simultaneously, but as dissociable entities, the probability of their remaining associated within a virtual "sea" of recipient-cell H-2 would be very low.

The rationale for experiments of the second type was as follows. If the association between viral and H-2 antigens resulted in altered-self, then priming of H-2 nonidentical mice

with fragments prepared from L-VAC and 3T3-VAC might possibly induce CTL with lytic specificity for virus + alloantigen. However, if these antigens were distinct from one another, the expected specificities of the CTL induced would be for alloantigen alone and virus + self. The assumption made here was that any existing complexes of altered-self expressed by the priming fragments would have been no more susceptible to degradation *in vivo* then either viral antigens or alloantigens alone, and therefore should have remained intact during the stage of antigen preparation.

The results of antigen-transfer experiments provided no evidence for the existence of altered-self. Lysis of membrane-hybrid target cells by virus-specific CTL of fragment-recipient H-2 haplotype was always greater than lysis achieved by CTL of fragment-donor haplotype (Table II). This occurred despite the fact that these same targets were highly susceptible to lysis by alloreactive CTL specific for H-2 antigens of the fragment-donor cells (Table I). Impressive lysis of membrane-hybrid targets by virus-specific CTL of fragment-donor haplotype could be demonstrated only when recipients of allogeneic fragments were productively infected with VAC (Table II). Under these conditions, surface membranes of recipient cells express large amounts of viral antigens, whereas their H-2 concentration appears to be decreased (35). Such conditions would favor the association, and therefore the recognition, of viral antigens with the transferred alloantigens.

Experiments in which mice were primed with VAC-bearing allogeneic membrane fragments also yielded results arguing against the formation of altered-self. In all cases, these animals responded as though they had been immunized separately with allogeneic membrane fragments and virus, exhibiting 1° CTL responses specific for H-2 antigens of the fragment-donor cells and for virus + self (Table III). Furthermore, *in vitro*, T cells from identically primed animals yielded potent virus-specific 2° CTL only when restimulated with VAC-bearing H-2 identical cells (Table IV). Virus-specific lysis could be differentiated clearly from allospecific lysis by the use of cold competing targets (Table V).

The patterns of allospecific and virus-specific CTL induction obtained by *in vivo* priming were duplicated when membrane fragments were used to induce 1° CTL *in vitro*. Again, virus-specific lysis was restricted to target cells sharing H-2 identity with the responding T cells (Table VI).

A potentially important point suggested by the antigen-transfer experiments is that the concentration of H-2 required for allospecific lysis (of membrane-hybrid targets) is less than that required for virus-specific lysis. For example, L cell recipients ($H\text{-}2^k$) of 3T3-VAC fragments ($H\text{-}2^d$) became highly susceptible to lysis by both anti-$H\text{-}2^d$ CTL and $H\text{-}2^k$-restricted anti-VAC CTL but only marginally lysable by $H\text{-}2^d$-restricted anti-VAC CTL (Table III). The basis for this apparent difference between allospecific and virus-specific CTL is unclear. One possibility may be that H-2 determinants are recognized allospecifically by receptors having high binding affinities, whereas recognition of these same determinants as self, and in conjunction with viral antigens, occurs through receptors having relatively low binding affinities (36). Alternatively, receptors that recognize H-2 may be present in greater numbers on alloreactive CTL.

The results of this study also bear on other more speculative aspects of CTL induction and recognition. The *in vitro* phenomenon of viral antigen-transfer may reflect similar occurrences *in vivo*. Virus replication, in general, has been shown to involve both the internal and external membranes of host cells, which, in many cases, eventually are destroyed. Conceivably, membrane fragments of such cells might continue to transfer viral antigens to uninfected "bystander" cells even after free virus particles were neutralized by antiviral antibody. If these bystanders were macrophages, CTL induction might be enhanced (See Table VI), whereas other types of cells in parenchymatous tissues might become the targets of CTL, thereby amplifying an ongoing immunopathologic process.

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