SYNTHETIC PHOSPHOLIPID VESICLES CONTAINING A PURIFIED VIRAL ANTIGEN AND CELL MEMBRANE PROTEINS STIMULATE THE DEVELOPMENT OF CYTOTOXIC T LYMPHOCYTES*

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The identification of the structures recognized by syngeneic cytotoxic T lymphocytes (CTL)[1] is complicated by the necessity for these cells to recognize, on the target cell surface, both antigen (Ag) and certain products of the major histocompatibility complex, rather than the Ag alone (1). To develop a general procedure for the identification of the Ag (such as those on virus infected or tumor cells), we have investigated the ability of synthetic phospholipid vesicles (liposomes), containing purified Ag, to specifically elicit the development of the corresponding CTL. We show here that CTL primed in vivo can be stimulated in a secondary response by liposomes containing both a single purified protein of vesicular stomatitis virus (VSV), the G or glycoprotein, and solubilized membrane proteins from cells of the same H-2 haplotype as the CTL and the VSV-infected target cells. Responder cells from ($H\text{-}2^d \times H\text{-}2^b$) hybrid mice ($d \times b$) and liposomes that contained G protein plus membrane proteins from cells of the parental H-2 haplotype, *d* or *b*, provided particularly striking evidence that CTL precursors were stimulated by intact liposomes, rather than by substances that were released from liposomes and subsequently adsorbed onto cells of the responder population.

Materials and Methods

Mice and Cultured Cells. H-2 haplotypes are in parentheses. BALB/cAnN (d) and H-2 congenic BALB.B (b) mice were bred at the Center for Cancer Research, Massachusetts Institute of Technology. (BALB/cAnN × BALB.B) F_1 hybrids were produced at Bowman

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[1] *Abbreviations used in this paper:* Ag, antigen(s); CTL, cytotoxic T lymphocytes; DMPC, dimyristoylphosphatidylcholine; G protein, purified glycoprotein of VSV; HLA, histocompatibility complex antigens; PBS, phosphate-buffered saline; PFU, plaque-forming unit(s); PMP, plasma membrane proteins; VSV, vesicular stomatitis virus.

Gray School of Medicine. The following cell lines were maintained in culture: P815 (d), a mastocytoma of DBA/2 origin, generously provided by M. Bevan, M.I.T.; EL-4 (b), a thymus lymphoma of C57BL/6 (B6) origin, kindly donated by H. Winn, Massachusetts General Hospital; MC57G (b), a macrophage-like cell line of B6 origin, kindly provided by Barbara Knowles, Wistar Institute; 3T3 fibroblasts were derived from BALB/c (d), BALB.B (b), and BALB.K (k) mice (2).

Viral and Cell Membrane Proteins. VSV and the virion glycoprotein (G protein) were produced and isolated as described (2, 3). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis the G protein migrated as a single band; its concentration was determined by the Lowry et al. method (4).

Plasma membranes were prepared as described (5). Briefly, ca. 6×10^9 cells were disrupted by gentle homogenization in 10 mM Tris (pH 7.8), 10 mM NaCl, 1 mM $MgCl_2$, and 1 mM phenymethylsulfonylfluoride. After low speed (400 *g*) centrifugation to remove nuclei and intact cells, crude membranes were obtained by centrifuging the supernate at 20,000 *g* for 0.5 h. The resulting pellet was resuspended in 40% sucrose in 10 mM Tris (pH 7.4), overlaid with 30% sucrose in 10 mM Tris (pH 7.4), and spun at 80,000 *g* for 18 h. The turbid interfacial band, containing plasma membrane (6), was removed and freed of sucrose by repeated centrifugations. The washed membranes, resuspended in PBS, were used immediately or frozen at −70°. The membranes were stirred overnight at 22°C with 0.5% Na deoxycholate, 0.45 M KCl, 10 mM Tris, pH 7.9; then, after centrifugation at 180,000 *g* for 1 h, the protein content of the supernate (plasma membrane proteins or PMP) was determined by measuring optical density, assuming 1 mg of protein/ml had an absorbance at 280 nm of 1.0. P815 and EL4 cells were used, respectively, as sources of PMP of H-2^d and H-2^b type.

Preparations of Phospholipid Vesicles. A modified procedure of Kagawa and Racker was used (7). Dimyristoylphosphatidylcholine (DMPC), purchased from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.), was used without further purification. Cholic acid, purchased from Sigma Chemical Co. (St. Louis, Mo.), was decolorized with activated charcoal and recrystallized twice from acetone. In a typical preparation, 0.6 mg DMPC, dried from chloroform under N_2, was suspended in 0.6 ml of cholic acid (4 mg/ml, pH 7.5) with a vortex mixer and was then sonicated for 15 min. After adding 10–20 μg of G protein (in 0.1 ml) and/or 300–400 μg of solubilized plasma membrane proteins (in 3–4 ml) the mixture was incubated for 1 h and then dialyzed overnight (all at room temperature) against 4 liters of 10 mM Tris, pH 7.5. To facilitate removal of deoxycholate, 20 ml (packed volume) of SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, Calif.) were added to the dialysis buffer. The liposomes that formed were packed by centrifugation (40,000 *g*), resuspended in 100 μl PBS, and sterilized under UV light (4 h, 15 cm from a Westinghouse sterilamp G15T8, Westinghouse Electric Corp., Pittsburgh, Pa.) before being added to spleen cell cultures. Through the use of ^{125}I-labeled G protein and ^{131}I-plasma membrane proteins (PMP), it was found that ~60% of the added G protein and ~30% of the added PMP were incorporated into the liposomes, whether G protein and PMP were incorporated into liposomes individually or together. The liposomes were shown by electron microscopy to range in diameter from 600 to 1,000 Å (D. Loh, unpublished material, 11) and to be unilamellar (11).

Immunization and Generation of Effector Cells. Mice were injected (primed) intravenously with $0.5–1.0 \times 10^7$ plaque-forming units (PFU) of plaque-purified VSV (not inactivated) in PBS. 5–10 wk later, 8×10^6 spleen cells, cleared of erythrocytes, were cultured in vitro with UV-inactivated VSV ($3–6 \times 10^7$ PFU before inactivation) or with liposomes containing one of the following: (*a*) G protein (ca 1.5 μg) plus PMP (10 μg), or (*b*) G protein alone (1.5 μg), or (*c*) PMP alone (10 μg), or (*d*) a mixture of (b) and (c). After 5 d, the cells were tested for ability to lyse ^{51}Cr-labeled VSV-infected target cells (2). Target cells were prepared by infecting P815 or MC57G cells or various congenic BALB 3T3 fibroblasts with VSV at a multiplicity of 20–25; 3.5 hrs later the cells were labeled with ^{51}Cr (8). 5×10^4 target cells and various numbers of effector cells were incubated for 6 h in 12 × 75-mm round-bottom plastic tubes (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) in a final vol of 400 μl. Controls for the effector cells in each assay included spleen cells from both primed and umprimed mice, cultured for 5 d in the absence of Ag (VSV or G protein or PMP).

Specific lysis resulting from the secondary stimulus was calculated as 100 (E-C/100-C) where

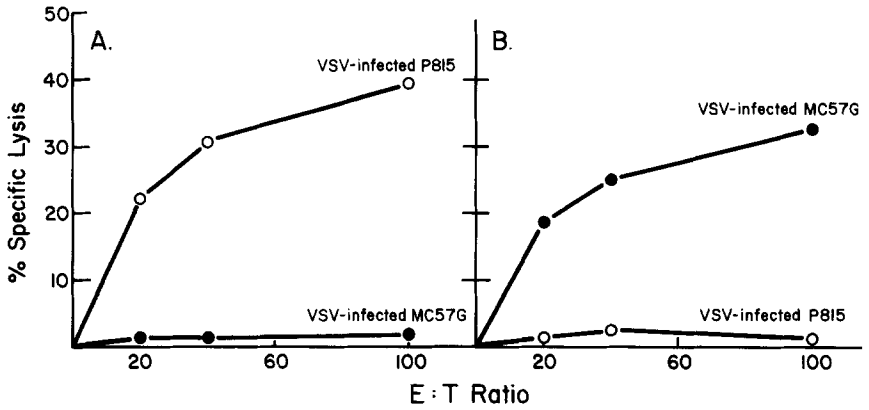

FIG. 1. H-2 restriction of the lytic activity of anti-VSV CTLs elicited by phospholipid vesicles. Target cells were ^{51}Cr-labeled VSV-infected P815 (H-2^d) or MC57G (H-2^b) cells. A. Secondary response by spleen cells from VSV-primed BALB/c (H-2^d) mice elicited in culture with vesicles containing VSV G protein and plasma membrane proteins from H-2^d cells (P815). B. Secondary response by spleen cells from VSV-primed BALB.B (H-2^b) mice elicited in culture with vesicles containing VSV G protein and plasma membrane proteins from H-2^b cells (EL-4).

E is the percentage of ^{51}Cr-released by primed and boosted spleen cells and C is the percentage of ^{51}Cr-released by primed, but not boosted, spleen cells. Values for C were typically 15–20%; in control assays with nonprimed and nonboosted spleen cells, 10–20% of target cell ^{51}Cr was usually released.

Media. The media used for cell culture and for cytotoxicity assays were RPMI-1640 supplemented as described (2).

Results and Discussion

A previous study of target cells infected with temperature-sensitive mutants of VSV showed that anti-VSV CTL lyse only target cells that have both the correct H-2 haplotype and G on the target cell surface (2). To determine whether purified G can elicit the formation of anti-VSV CTLs, we examined the response of spleen cells from VSV-primed mice to liposomes containing G. Spleen cells removed 6 d after a single intravenous injection of VSV have considerable CTL activity; however, 5–6 weeks later CTL activity is no longer detectable. To stimulate a secondary CTL response, spleen cells taken 6–8 w after one i.v. injection of VSV were incubated for 5 d with liposomes containing purified G protein, with or without cell membrane proteins; the spleen cells were then tested on VSV-infected target cells with various H-2 haplotypes.

As is shown in Fig. 1, when primed spleen cells from BALB/c (H-2^d) mice were incubated with liposomes that contained both purified G and membrane proteins from H-2^d cells, they elicited CTLs that were VSV-specific and H-2 restricted: they lysed VSV-infected H-2^d cells (P815) but neither VSV-infected H-2^b cells (MC57G) (Fig. 1 A) nor uninfected P815 cells (not shown). Parallel results were obtained with primed spleen cells from congenic BALB.B (H-2^b) mice: the CTL they formed in response to liposomes having G and PMP from H-2^b cells lysed VSV-infected H-2^b cells (MC57G), not VSV-infected H-2^d cells (P815) (Fig. 1 B).

The triggering of the secondary CTL response also required a match between the H-2 haplotype of the primed spleen cells and the cells whose membrane proteins were included, with G, in the liposomes. As is shown in Fig. 2, anti-VSV CTLs were elicited

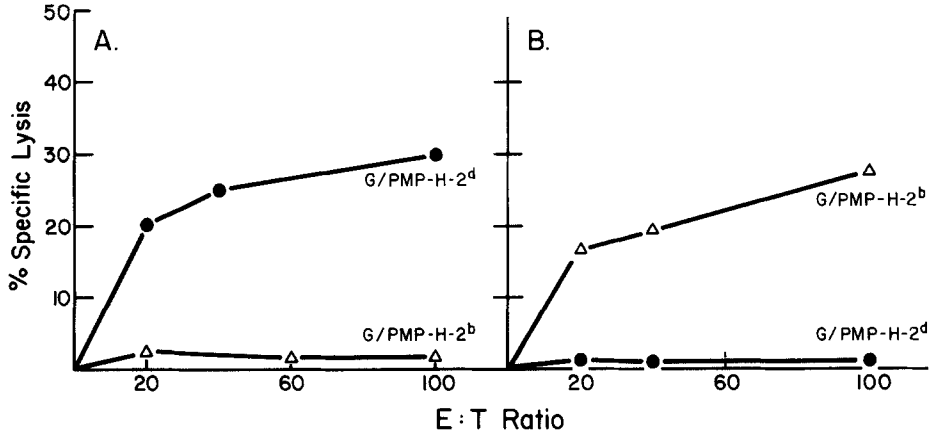

FIG. 2. H-2 restriction in the generation of anti-VSV CTLs by incubating spleen cells from VSV-primed BALB/c (H-2^d) and BALB.B (H-2^b) mice with phospholipid vesicles containing VSV G protein and plasma membrane proteins from uninfected P815 (H-2^d) cells or EL-4 (H-2^b) cells, designated G/PMP-H-2^d (●) and G/PMP-H-2^b (△), respectively. A. The primed mice were BALB/c and the target cells were ^{51}Cr-labeled VSV-infected P815 cells (H-2^d). B. The primed mice were BALB.B and the target cells were ^{51}Cr-labeled VSV-infected MC57G cells (H-2^b).

in primed spleen cells from H-2^d mice (BALB/c) by liposomes with G + PMP from H-2^d cells, not by liposomes with G + PMP from H-2^b cells, and the reverse was observed with primed spleen cells from H-2^b mice (BALB.B). These results strongly imply that CTL precursors were stimulated by intact liposomes: if G protein was released and then elicited CTL, the H-2 haplotype of the cells whose membrane proteins were incorporated into liposomes would have been irrelevant.

The effectiveness of intact liposomes was brought out especially forceably with primed spleen cells from VSV-infected (BALB/c × BALB.B)F_1 hybrid mice, for simplicity called *d* × *b*. Anti-VSV CTLs could be elicited in these *d* × *b* spleen cells with G-bearing liposomes that contained membrane proteins from cells with either parental H-2 haplotype, H-2^b or H-2^d (Table I). There is considerable evidence that F_1 hybrids form two sets of Ag-specific CTLs, one for Ag + H-2 of one parent and the other for Ag + H-2 of the other parent (9). In accord with this evidence, the *d* × *b* CTL elicited with liposomes that contained G + PMP from H-2^d cells lysed VSV-infected target cells with the H-2^d haplotype, not targets with the H-2^b haplotype, whereas the *d* × *b* CTL elicited with G + H-2^b membrane proteins lysed only VSV-infected targets with the H-2^b haplotype (Table I). Again, if *d* × *b* CTL were elicited by the G protein released from disintegrated liposomes it would be expected that the resulting anti-VSV CTLs would have lysed infected H-2^d and H-2^b targets, regardless of which liposomes elicited the killer cells. It has been proposed that T cells recognize not intact Ag molecules but processed Ag fragments on macrophages (e.g., reference 10). The findings in Table I suggest, however, that Ag processing is not necessary, at least for the specific stimulation of secondary CTL responses.

In contrast to the stimulator activity of liposomes with G + PMP (from cells of the correct H-2 haplotype), liposomes with G alone, or with the PMP alone, were ineffective. A mixture of the G-alone and the PMP-alone liposomes was also ineffective (Fig. 3). This means that for effective interaction with CTL precursors the G and H-

TABLE 1

Spleen Cells from Primed (H-2^d × H-2^b) Hybrid Mice Respond in Culture to Phospholipid Vesicles Containing G Glycoprotein and Plasma Membrane Proteins from Uninfected Cells of Parental H-2 Haplotype

Liposomes contain	Percent specific lysis of VSV-infected target cells*				
			3T3 Fibroblasts		
	P815 (H-2^d)	MC57G (H-2^b)	BALB/c (H-2^d)	BALB.B (H-2^b)	BALB.K‡ (H-2^k)
G alone	4.8	4.6	5.0	5.2	−2.4
G + membrane proteins from H-2^d cells	**46.7**	3.5	**18.8**	5.1	0.3
G + membrane proteins from H-2^b cells	5.0	**43.9**	2.7	**24.0**	0.2

* Additional controls (A. H. Hale, unpublished material) were carried out by incubating spleen cells from VSV-primed (H-2^d × H-2^b) F_1 mice with the following mixtures of liposomes, containing G protein and/or plasma membrane proteins from H-2^d or H-2^b cells (designated as PMP-H-2^d or PMP-H-2^b, respectively):

(No. 1) (G + PMP-H-2^b) liposomes mixed with (G + PMP-H-2^d) liposomes
(No. 2) (G + PMP-H-2^b) liposomes mixed with (PMP-H-2^d) liposomes
(No. 3) (G + PMP-H-2^d) liposomes mixed with (PMP-H-2^b) liposomes.

Percent specific lysis of VSV-infected H-2^d and H-2^b target cells (P815 and MC57G, respectively) were as follows (at E:T ratio 100:1):

mixture no. 1—**26.8, 37.3**
mixture no. 2—6.7, **41.8**
mixture no. 3—**31.8,** 10.2.

In these controls the liposomes were prepared with egg lecithin-cholesterol (70:30, wt/wt) and the indicated proteins in 0.2% deoxycholate; vortex mixing was used to disperse the lipids and dialysis to remove the deoxycholate.

‡ When tested separately these targets were shown to be specifically lysed (38.4%) by primary response CTLs spleen cells taken from BALB.K mice 6 d after an intravenous injection of 2 × 10^7 PFU of VSV.

2 molecules had to be in the same liposome, or closer together than 600–1,000 Å, the diameter of the unilamellar vesicles used in this study (11, and also A. H. Ross and H. G. Khorana, unpublished results).

For comparison with liposomes, UV-inactivated VSV and soluble G protein, not incorporated into vesicles, were also tested on VSV-primed spleen cells. The inactivated virus regularly elicited as much CTL activity as active liposomes (data not shown).[2] In contrast, G protein, added at 5, 10, 50, and 100 μg/well, failed to elicit a significant response (data not shown); however, in a few trials, where lysis elicited by liposomes (with G and PMP from cells with the correct H-2 haplotype) was relatively high (e.g., 60–70% specific lysis), some cytolytic activity was elicited by soluble G protein, but the response was low (ca. 10–15%) and not reproducible.

Two previous studies showed that Ag-containing liposomes can elicit CTL. Purified

[2] In many trials stimulation of the primed spleen cells by the inactivated virus also resulted in a low level of lysis of uninfected P815 cells, especially when the activity elicited againt VSV-infected P815 was high (e.g., 60% specific lysis). A definite explanation for the low-level, apparently nonspecific cytotoxic activity is not available (but see ref. 21 for one suggestion).

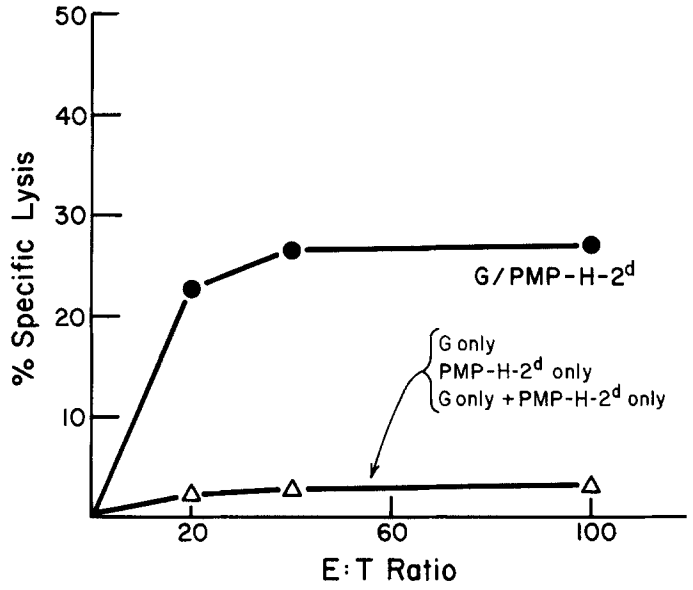

FIG. 3. Stimulation of CTL activity in spleen cells from VSV-primed BALB/c (H-2^d) mice by variously prepared liposomes: ●, vesicles with VSV G protein + membrane proteins from P815 (H-2^d) cells [G/PMP-H-2^d]. Δ, vesicles with G protein only, or only with P815 membrane proteins, or a mixture of the two kinds of vesicles (G only and PMP only).

histocompatibility leukocyte antigens (HLA) in liposomes were used by Englehard et al. (12) to stimulate the development of mouse CTL that specifically lysed human cells of the correct HLA type, and Finberg, et al. (13) used liposomes containing a mixture of Sendai virus Ags (plus cell membrane proteins of the correct H-2 haplotype) to elicit H-2 restricted CTLs that lysed Sendai virus-infected target cells. However, Sendai virus proteins cause cells to fuse (14), and probably liposomes and cells to fuse, whereas VSV and purified G protein have no fusing activity (15). It is not clear, therefore, that Sendai-liposomes and the VSV-liposomes described here function in precisely the same manner.

Even without being incorporated into lipid vesicles, some isolated proteins can apparently elicit H-2 restricted CTLs (16–20). It is probable that these proteins bind to cells of the responder population and that the bound protein, through proximity with the appropriate H-2 encoded surface molecules on the same cells, is effectively presented to CTL precursors. Alternatively, it has been suggested that the soluble proteins (bound or unbound) might stimulate T helpers to secrete mediators that nonspecifically trigger the differentiation of any CTL precursors in the responder population (21). In the present study, however, there was a striking difference between soluble G and the G that was incorporated (along with cell membrane proteins) into liposomes: whereas 1.5 μg of G in liposomes (with H-2 proteins) consistently elicited secondary CTL (Figs. 1–3), soluble G, in amounts up to 100 μg was generally ineffective.

The identity of the Ags (and antigenic determinants) that determine the specificity of CTL and other T cells has been difficult to establish. With the finding that purified protein in intact liposomes (along with the appropriate H-2 products) can specifically

elicit secondary CTL it may be possible to define unambiguously not only the Ag molecules but the antigenic determinants that are recognized by the T cells.

Summary

Synthetic phospholipid vesicles (liposomes) containing the purified glycoprotein (G) of vesicular stomatitis virus (VSV) and solubilized membrane proteins from cells of the appropriate H-2 haplotype elicited H-2-restricted cytotoxic T lymphocytes (CTL) that lysed VSV-infected target cells. The CTL were elicited by intact liposomes, not by released components. Thus, when spleen cells from VSV-primed H-2^d × H-2^b hybrid mice were stimulated with liposomes having G protein + membrane proteins from cells with one of the parental H-2 haplotypes, the resulting CTL lysed only VSV-infected target cells with that parent's H-2 type. This result argues against the view that T cells in general recognize only processed antigenic fragments on macrophages. Moreover, liposomes were only effective when G protein and cell membrane proteins were included in the same vesicles. This result suggests that for effective interaction with CTL precursors the antigen (G protein) and products of the H-2 complex must be closer to each other than 600–1,000 Å, the diameter of the lipid vesicles used in this study.

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References

1. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* **29:**89.
2. Hale, A. H., O. N. Witte, D. Baltimore, and H. N. Eisen. 1978. Vesicular stomatitis virus glycoprotein is necessary for H-2 restricted lysis of infected cells by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **75:**970.
3. Kelley, M. J., E. V. Emerson, and R. R. Wagner. 1972. The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. *J. Virol.* **10:**1231.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **193:**265.
5. Allen, D., and M. J. Crumpton. 1970. Preparation and characterization of the plasma membrane of pig lymphocytes. *Biochem. J.* **120:**133.
6. Meissner, G., and S. Fleischer. 1974. Dissociation and reconstitution of functional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **249:**302.
7. Kagawa, Y., and E. Racker. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **246:**5477.
8. Cerottini, J. C., and K. T. Brunner. 1974. Cell-mediated cytotoxicity. *Adv. Immunol.* **18:**67.
9. Bevan, M. 1978. In radiation chimeras host H-2 antigens determine the immune responsiveness of donor cytotoxic cells. *Nature (Lond.)* **269:**417.
10. Benacerraf, B. 1978. A hypothesis to relate the specificity of T lymphocytes and the activity of I-region specific Ir genes in macrophages and B lymphocytes. *J. Immunol.* **120:**1809.
11. Chamberlain, B. K., Y. Nozaki, C. Tanford, and R. E. Webster. 1978. Association of the major coat protein of fd bacteriophage with phospholipid vesicles. *Biochim. Biophys. Acta.* **510:**18.
12. Engelhard, V. H., J. L. Strominger, M. Mescher, and S. Burakoff. 1978. Induction of secondary cytotoxic T lymphocytes by purified HLA-A and HLA-B antigens reconstituted

into phospholipid vesicles. *Proc. Natl. Acad. Sci. U. S. A.* **75:**5688.

13. Finberg, R., M. Mescher, and S. J. Burakoff. 1978. The induction of virus-specific cytotoxic T lymphocytes with solubilized viral and membrane proteins. *J. Exp. Med.* **148:**1620.
14. Gething, M.-J., U. Koszinowski, and M. Waterfield. 1978. Fusion of Sendai virus with the target cell membrane is required for T cell cytotoxicity. *Nature (Lond.).* **274:**689.
15. Fan, D. P., and B. M. Sefton. 1978. The entry into host cells of Sindbis virus, vesicular stomatitis virus, and Sendai virus. *Cell.* **15:**985.
16. Mescher, M., L. Sherman, F. Lemonnier, and S. Burakoff. 1978. The induction of secondary cytolytic T lymphocytes by solubilized membrane proteins. *J. Exp. Med.* **147:**946.
17. Alaba, O., and L. W. Law. 1978. Secondary induction of cytotoxic T lymphocytes with solubilized syngeneic tumor cell plasma membranes. *J. Exp. Med.* **148:**1435.
18. Braciale, T. J. 1979. Specificity of cytotoxic T cells directed to influenza virus hemagglutinin. *J. Exp. Med.* **149:**856.
19. Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Courtneidge, and J. J. Skehel. 1977. Cytotoxic T cells to type A influenza virus: viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* **7:**630.
20. Schmitt-Verhulst, A.-M., C. B. Pettinelli, P. A. Henkart, J. K. Lunney, and G. M. Shearer. 1978. H-2-restricted cytotoxic effectors generated in vitro by the addition of trinitrophenyl-conjugated soluble proteins. *J. Exp. Med.* **147:**352.
21. Henney, C. S. 1979. The "triggering" of cytotoxic cell differentiation in secondary culture by subcellular antigens. *J. Immunol.* **122:**2134.