

RECOGNITION OF VIRAL GLYCOPROTEINS
BY INFLUENZA A-SPECIFIC CROSS-REACTIVE
CYTOLYTIC T LYMPHOCYTES*

BY ULRICH H. KOSZINOWSKI, HAYMISH ALLEN, MARY-JANE GETHING,
MICHAEL D. WATERFIELD, AND HANS-DIETER KLENK

From the Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Federal Republic of Germany; the Protein Chemistry Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England; and the Institut für Virologie der Justus Liebig Universität, D-6300 Giessen/Lahn, Federal Republic of Germany

After infection with unrelated viruses such as paramyxoviruses, orthomyxoviruses, poxviruses, and rhabdoviruses (1) mice generate cytolytic T lymphocytes (CTL)¹ that possess selective specificity for the sensitizing virus. However, after infection with different subtypes of serologically distinct influenza A viruses (2) at least more than one population of CTL can be demonstrated. Both cross-reactive and subtype-specific lymphocytes can be distinguished (3-12).

These cross-reactive effector T cells may lack the discriminatory capacity for influenza antigens shown by the B cell products, or they may recognize a shared antigenic determinant. Because the question of the nature of antigens recognized by T cells, as well as the structure and composition of the T cell receptor, is not yet clear, the orthomyxoviruses offer a biological relevant system for the analysis of T cell specificity.

The subtypes of influenza A viruses differ with respect to the antigenicity of the envelope glycoproteins, hemagglutinin (HA) and neuraminidase (N). HA and N are inserted into the plasma membrane of cells during the budding process that results in virus release from the infected cells (13, 14). The internal virion components, the matrix (M) protein and the ribonucleoprotein (NP), of all influenza A viruses seem to be closely related antigenically. The cell-membrane expression of antigens is a prerequisite for T cell recognition (15-17). The work of several authors has therefore centered on the question of membrane expression of these antigens. M protein has now been detected on the cell membrane, and, consequently, it has been claimed that the cross-reactive T cell may recognize this protein (8, 18-22).

This study aims to clarify the role of M protein in the CTL response. Target cells were prepared by two different methods. First by abortive infection of L-929 cells

* Supported by the Deutsche Forschungsgemeinschaft 571/5 and 571/7.

¹ *Abbreviations used in this paper:* Chol, cholesterol; CTL, cytolytic T lymphocytes; EM, electron microscopy; FPV, fowl plague virus; HA, hemagglutinin; HAU, hemagglutinating unit(s); M, matrix; MOI, multiplicity of infection; N, neuraminidase; NP, ribonucleoprotein; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFU, plaque-forming unit(s); PMSF, phenylmethylsulfonylfluoride; PS, L-phosphatidyl-L-serine; SDS, sodium dodecyl sulfate; SV, Sendai virus; TN, Tris-buffered saline; TRY-SV, trypsin-inactivated SV.

with an avian influenza A strain that expresses only the viral envelope glycoproteins, HA and N, in the plasma membrane (23). Second, the HA and N glycoprotein of the human influenza virus A/Victoria were separated from the other virion components and reconstituted into artificial lipid bilayers (24, 25) to give vesicles free of M protein and NP. Target cells were then prepared by incubating these vesicles with cells in the presence of fusion-active Sendai virus (26). Various influenza A-specific CTL were tested against both types of target cells. The results showed a similar pattern of cross-reactivity with that previously observed after infection of cells with different human influenza A virus subtypes. Thus target antigens recognized by cross-reactive CTL are not carried by M protein but by the glycoproteins HA or N in the plasma membrane.

Materials and Methods

Viruses. Sendai virus; the influenza viruses A/Asia/M/1468 (H2N2), A/PR/8/34 (H0N1), A/Victoria/3/75 (H3N2), and B/Mass/71; and the avian influenza strains A/FPV/Rostock (Hav1/N1) and A/Tur/Canada/63 (Hav6 Neq2) were used. Viruses were grown in the allantoic cavity of 11-d-old embryonated chicken eggs, and virus stocks were stored in the allantoic fluid at -80°C . Plaque assays for the determination of plaque-forming units (PFU), titration of hemagglutinating units (HAU), N and hemolytic activities were carried out by established procedures (15).

Purification of Sendai virus (SV), inactivation with β -propiolactone, and digestion of trypsin-inactivated SV (TRY-SV) to destroy the functional activity of the fusion protein were done as described previously (15, 17).

Preparation of Influenza A Glycoprotein and Reconstitution into Artificial Vesicles. Purified influenza A/Victoria was a gift from Searle Laboratories, London. Virus protein was determined by the Lowry method using bovine serum albumin standards. Influenza A/Victoria virus (10 mg/ml) was suspended in phosphate-buffered saline (PBS), without Mg or Ca, that contained 2% Empigen BB (Albright & Wilson Ltd., Detergents and Chemical Group, Whitehaven, England) and 1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., London). Preparation of tritium-labeled virus was carried out according to the method of Critchley (27). Disruption of virus and solubilization of glycoproteins was achieved by three separate 15-s sonications at setting 3 with a Branson sonicator probe (model 2530-IA, Branson Sonic Power Co., Danbury, Conn.) at 4°C . The disrupted virus was dialyzed overnight against 1 M Tris buffer (pH 6.8) that contained 2% Empigen BB, 1 mM PMSF, and 100 mM sodium azide. The preparation (25 mg unlabeled virus in 4 ml and 200 μg of tritium-labeled virus) was then layered onto a sucrose gradient that consisted of three zones similar to that described by Helenius et al. (24): A 2-ml 10% sucrose zone that contained 2% Empigen BB on top, a 30-ml linear 15–30% sucrose gradient that contained 1% Empigen BB in the middle, and a 2-ml 50% (wt/vol) sucrose cushion in Tris-buffered saline (TN) buffer (pH 7.4) at the bottom.

After centrifugation (40 h, 4°C , 25,000 rpm) in a Beckman SW 27 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.), fractions were collected from the bottom and assayed for radioactivity and protein concentration. The protein-containing fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (28) with 10% gels. Two-dimensional gel electrophoresis was carried out according to the method of Wang and Richards (29). Fractions that contained only the envelope glycoproteins were used for reconstitution. Reconstitution was done according to a protocol developed for reconstitution of SV envelope proteins (M.-J. Gething. Unpublished observations.): The lipids L-phosphatidyl-L-serine (PS) (10 mg/ml) (Sigma Chemical Co.); phosphatidylethanolamine (PE) (1 mg/ml) (BDH Chemicals Ltd., Poole, England); phosphatidylcholine (PC) (10 mg/ml) (Sigma Chemical Co.); and cholesterol (Chol) (10 mg/ml) (Sigma Chemical Co.) that were suspended in chloroform:methanol 95:5 (vol/vol) were used. Lipids were mixed in the ratio of 2 PE:1 PS:1 PC:1 Chol in 30-ml tubes, and the chloroform and methanol were evaporated under nitrogen, washed twice with the same volume of diethylether, and dried under nitrogen to obtain a thin lipid film. The film was dissolved in TN buffer that contained

1 mM PMSF, 2 mM CaCl₂, 2 mM MgCl₂, and 1% Empigen BB by heating for 30 s at 100°C. The detergent-purified HA and N viral glycoproteins in TN buffer that contained 1% Empigen BB were added. Reconstitution was achieved by detergent dilution (24). The detergent was diluted by stepwise addition of TN buffer over a period of 5 h. A control preparation of HA and N was also made without lipid. The reconstituted HA and N vesicles were concentrated by centrifugation (15 h, 20°C, 38,000 rpm, 8- × 50-ml MSE Rotor, MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England). The pellet was resuspended in TN buffer and analyzed by polyacrylamide gel electrophoresis and electron microscopy.

Mice. Female C3H (H-2K), CBA/J (H-2K), C3HOH (H-2^O), and DBA/2/Den (H-2^d) mice were bred at the German Cancer Research Center, Heidelberg, Federal Republic of Germany or purchased from Ry Bomholtgard, Copenhagen, Denmark, and used at 8–12 wk of age. Mice were immunized by single inoculation of 200–400 HAU i.p. of virus. Effector-cell generation by secondary challenge with homologous virus in vitro was done as described previously (15, 17, 30, 31). Spleen cells from mice primed 3–6 wk before testing were cultured in the presence of stimulator cells at a ratio of 10:1 for 5–7 d in RPMI-1640 medium supplemented with antibiotics, 2 × 10⁻⁵ M 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Stimulator cells were prepared by incubation of syngeneic spleen cells with 1–10 HAU of virus/10⁶ cells for 60 min followed by a threefold washing of cells.

Cytolytic Assay. P-815 (H-2^d) and L-929 (H-2^k) cells were grown in tissue culture. For target-cell formation with influenza viruses, 10⁶ cells were incubated either with 200 HAU of virus or with preparations that contained known amounts of PFU. After 60 min, virus adsorption cells were washed three times and labeled with ⁵¹Cr for a further 3 h. Target cells prepared with inactivated SV and TRY-SV were obtained as described before (17). Preparation of target cells with vesicles was carried out as previously described (26): Influenza vesicles were bound to cells by incubation of 10⁶ cells with 30 μg of vesicles for 4 h. Fusion of influenza A vesicles that contained HA and N was achieved by coinubation of cells with 30 μg of vesicles and 10 μg of fusion-active SV for 4 h. A control for fusion was carried out with the same amount of TRY-SV. In the cytolytic assay, target cells were incubated with CTL at four effector:target ratios. After a 4-h incubation at 37°C in 5% CO₂ atmosphere, supernates were harvested and the specific release was determined as percent specific ⁵¹Cr release = (release by effector cells – spontaneous release/maximal release – spontaneous release) × 100. Spontaneous release was determined from target cells incubated with medium only, and maximal release from target cells incubated with 2% SDS in PBS. All values are the mean percent specific ⁵¹Cr release of at least three wells.

Results

Abortively Infected Targets Devoid of M Protein Are Lysed by Cross-Reactive CTL. In L-929 cells infected with influenza A/fowl plague virus (FPV) the synthesis of M protein does not occur, although the virus-specific glycoproteins are synthesized and incorporated into the plasma membrane (23). This system was used to determine the role of the M protein as an antigen recognized by cross-reactive influenza A strain-specific CTL. L-929 target cells infected with 10 PFU/cell of A/FPV were incubated with secondary CTL of CBA mice sensitized in vivo and restimulated in vitro with FPV, A/Asia, PR/8, A/Turkey, or B/Mass. As expected, lysis was seen after addition of effector cells that had specificity for FPV (Fig. 1). However, the all-influenza-type-A-primed effector cells in addition to the effect on homologous targets also lysed the L-929 target cells infected with FPV. Only effector cells with specificity for B/Mass failed to lyse the L-929 FPV targets. This pattern of cross-reactivity that has been observed by other authors (3, 5, 7, 9) was also found when influenza A target cells that lacked M protein were used.

To exclude involvement of M protein from the virions used for cell infection, the following experiment was carried out: L-929 cells were infected with FPV at a

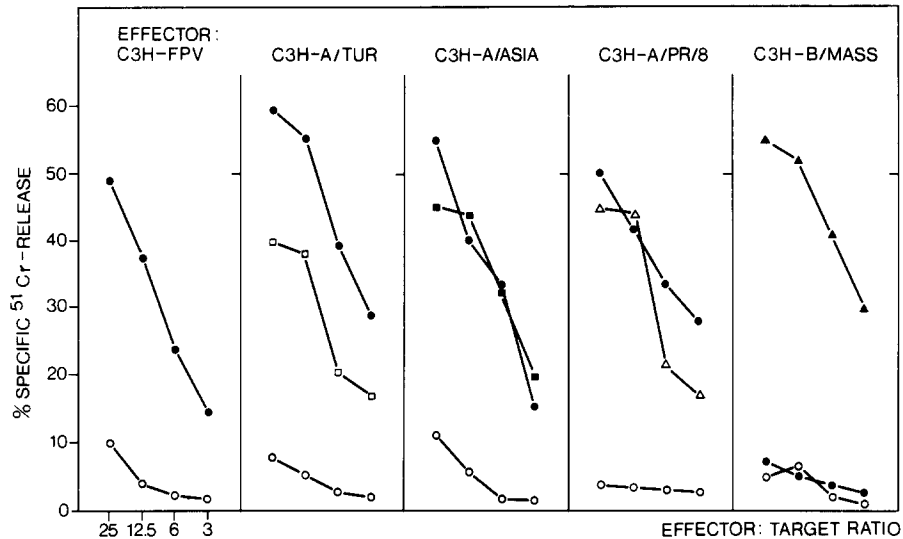


FIG. 1. Effect of influenza subtype-specific CTL on L-929 target cells abortively infected with 10 PFU/FPV cell. CTL were primed and restimulated with the homologous virus strain and tested on target cells infected with the sensitizing virus or with FPV. Target cells: (○) noninfected L-929; (●) L-929 infected with A/FPV; (□) L-929 infected with A/Turkey; (■) L-929 infected with A/Asia; (△) L-929 infected with A/PR/8; (▲) L-929 infected with B/Mass.

multiplicity of infection (MOI) of 0.01–100 PFU/cell and tested after a 4-h or overnight incubation after infection. Effector cells were secondary C3H.OH CTL with specificity for A/Asia. Fig. 2 shows that the same cross-reactivity pattern was observed at the MOI of 1 PFU/cell, which is too low to obtain lysable target cells after 4 h of infection, and 16 h of synthesis of viral glycoproteins is required for T cell-mediated lysis. Therefore, the M protein transported into the target cell during the process of penetration can not explain the cross-reactivity observed.

Reconstitution of Membrane Glycoproteins HA and N in Lipid Vesicles. Influenza A/Victoria virus was solubilized by sonication in detergent. The solubilized virus was centrifuged into a sucrose gradient in detergent. The spike proteins, HA and N, were recovered as a broad band in the upper part of the gradient; whereas NP was found in the bottom fractions and M protein and lipids at the top of the gradient. The fractions were analyzed by polyacrylamide gel electrophoresis, and the composition of the fractions used for reconstitution is shown in Fig. 3A and B. It is shown in two-dimensional gel that the high molecular proteins on the top of the gels contain trimers and dimers of HA as well as N, but no NP or M protein.

Lipid-free HA and N will not interact with cells to produce lysable targets (26), presumably because these integral viral membrane proteins are no longer correctly orientated in a lipid bilayer. To mimic the normal mode of presentation of these glycoproteins, the HA and N were reconstituted in lipid vesicles and integrated into the target cell membrane obtained by fusion.

The lipids PE, PS, PC, and Chol were solubilized in the detergent-solubilized proteins. The detergent concentration was reduced ~50-fold by stepwise dilution. The reconstituted material was analyzed by electron microscopy. The glycoproteins were

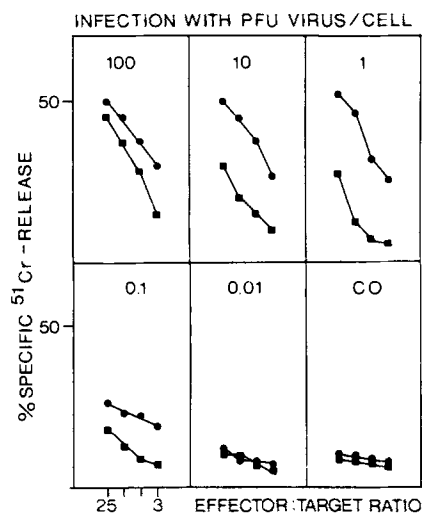


FIG. 2. Effect of virus concentration and viral protein synthesis on antigen formation recognized by cross-reactive CTL. Effector cells were C3H.OH lymphocytes primed in vivo and restimulated in vitro with A/Asia. Target cells were prepared by incubation of L-929 cells with different PFU/cell (number of PFU shown in each box) for (■) 4 h or (●) 16 h. CO, uninfected targets.

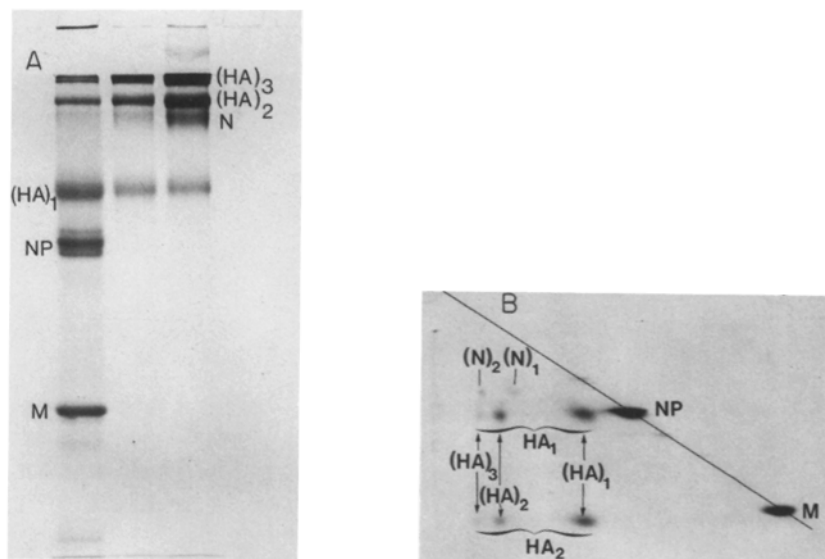


FIG. 3. (A) Polyacrylamide gel electrophoresis in SDS under nonreduced conditions of A/Victoria virus proteins, after gradient centrifugation and after reconstitution. Left lane: A/Victoria virus; middle lane: fraction with peak radioactivity and glycoprotein concentration; right lane: reconstituted preparation. (B) Two-dimensional gel of A/Victoria virus proteins. First dimension: Laemmli tube gel (5% stack, 10% running gels). Second dimension: under reduced conditions performed according to Wang and Richards (29). After splitting by reductive cleavage during the initial phase of the second-dimension slab gel, the molecule complexes are identified by their off-diagonal position on the final pattern. (HA)₁, HA monomers; (HA)₂, HA dimers; (HA)₃, HA trimers.

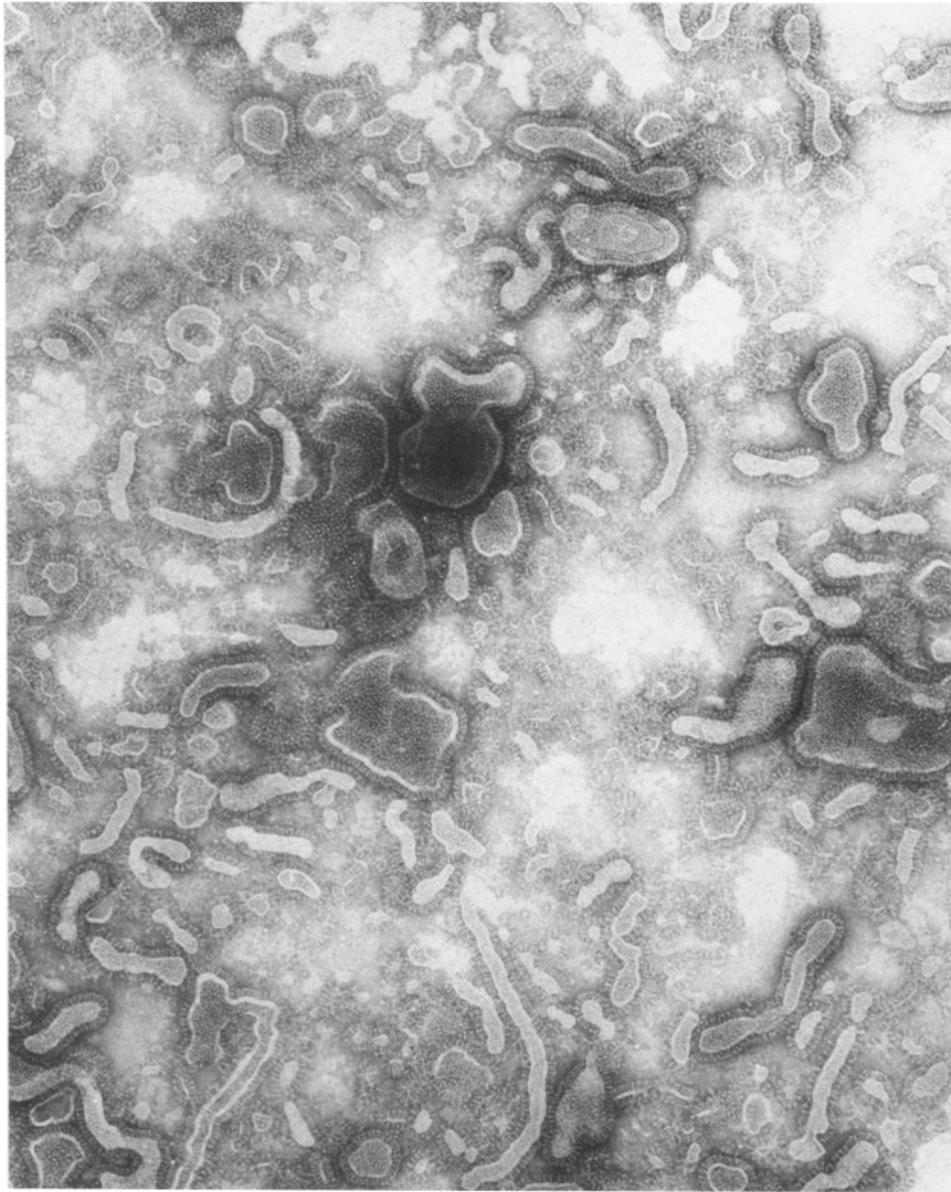


FIG. 4. Electronmicroscopy of reconstituted vesicles. $\times 120,000$.

observed as distinct spikes, the majority of which were associated with unilamellar vesicles that ranged in size from 10 to 50 nM (Fig. 4). Some vesicles were round or oval, whereas others were extended and irregular but enclosed by a lipid bilayer. A background of what are probably free spikes or rosettes of lipid-free spikes was also seen. The packing of spikes was similar to that observed on virus particles and it appeared that the spikes were orientated so that they projected outwards from the lipid bilayer. This outward orientation is supported by the observation that reconstituted vesicles showed 10- to 100-fold greater hemagglutinating activity than glyco-

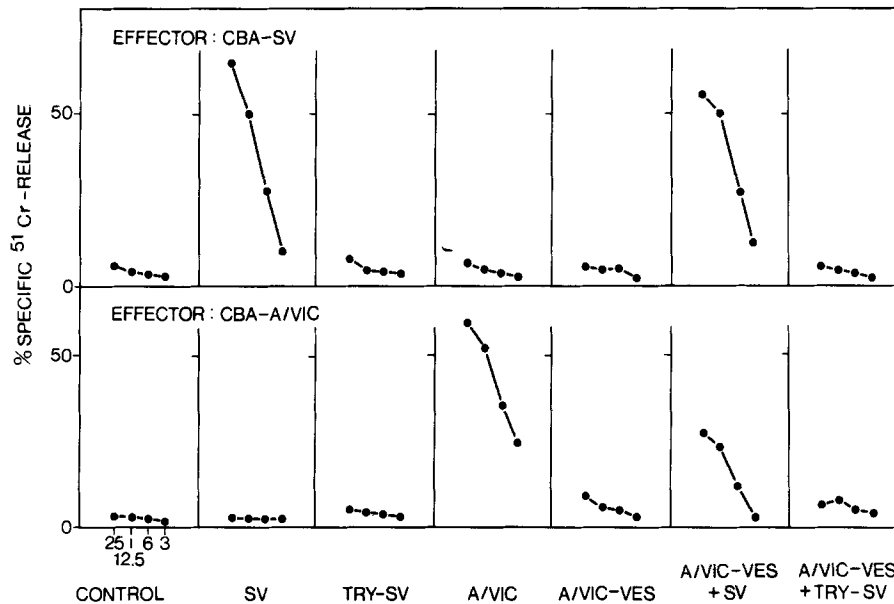


FIG. 5. Target cell formation with influenza envelope proteins reconstituted into lipid vesicles. Target cells were prepared by incubation of 10^6 L-929 cells with $10 \mu\text{g}$ β -propiolactone-inactivated SV, with $10 \mu\text{g}$ SV with enzymatically inactivated fusion protein (TRY-SV), 100 HAU influenza A/Victoria, $30 \mu\text{g}$ reconstituted envelope proteins of A/Victoria-virus vesicles (A/VIC-VES), or with mixtures of reconstituted vesicles with SV preparations. Incubation time of cells with virus was 4 h at 37°C . Targets were incubated with effector cells with specificity for either SV or influenza A/Victoria (A/VIC).

proteins that were carried through the reconstitution procedure in the absence of lipid. Such preparations contained rosettes and free glycoprotein spikes with no sign of vesicle formation (data not shown).

Target-Cell Formation with Influenza A/Victoria Glycoproteins Incorporated into Vesicles. Although vesicles that contain HA and N attach to target cells, fusion does not seem to occur. Thus we have used a recently developed method to prepare target cells with reconstituted Semliki forest virus membrane vesicles (26). Influenza A/Victoria vesicles were incubated with ^{51}Cr -labeled L-929 cells in the absence or presence of noninfectious β -propiolactone-treated, fusion-active SV or TRY-SV that was no longer capable of causing fusion. As control targets, noninfected L-929 cells, L-929 cells infected with influenza A/Victoria, or L-929 cells incubated with the two preparations of SV were used. Antigen-specific T cell-mediated cytotoxicity was tested by the addition to these targets of either SV-specific CTL or influenza A/Victoria-specific CTL (Fig. 5). Target cells prepared with TRY-SV were not susceptible to lysis, presumably as a result of the loss of fusion activity (17). Similarly, the targets incubated with influenza A/Victoria vesicles alone or together with TRY-SV showed a low level of lysis, which indicated that the mere presence of SV antigens is not sufficient to form influenza-specific target cells with vesicles. However, target cells incubated with the vesicles and fusion-active SV were lysed by both the influenza-specific CTL and the SV-specific CTL. The specificity of influenza A/Victoria CTL in this reaction is selectively directed to influenza A/Victoria antigens, and there is no evidence to support the idea that SV antigens are recognized by these effector cells.

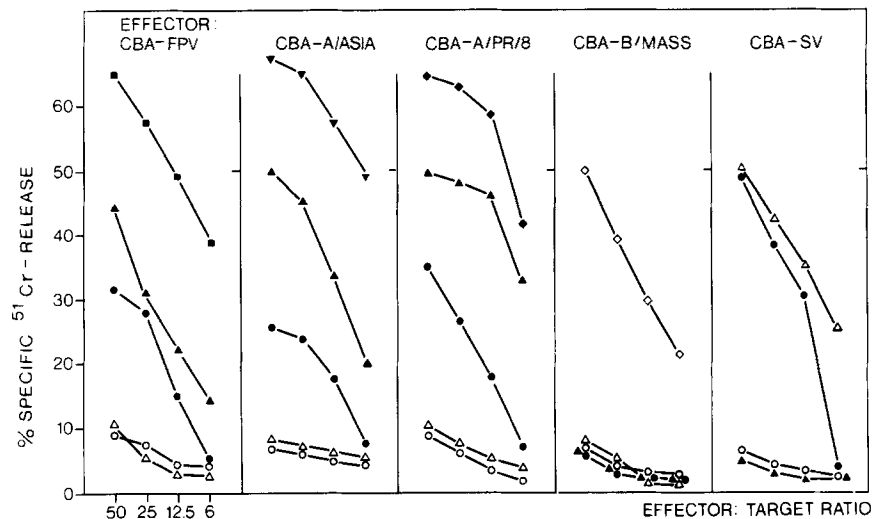


FIG. 6. Cytolytic activity of various influenza- and V-specific CTL on artificial target cells prepared with reconstituted virosomes. Target L-929 cells incubated with (●) A/Victoria-vesicles and SV (Fig. 5 legend); (▲) A/Victoria; (■) FPV; (▼) A/Asia; (◆) A/PR/8; (◇) B/Mass.; (△) SV; (○) noninfected L-929 control.

Cross-Reactive CTL Specific for Different Subtypes of Influenza A Recognize Antigens of the Influenza A/Victoria Envelope Proteins. Having established a method to test target cells prepared with the envelope proteins, a variety of effector cells specific for different influenza subtypes were tested. CTL with specificity for FPV, A/Asia, A/PR8, A/Victoria, and B/Mass. were tested on targets infected with homologous or heterologous virus or on targets prepared with reconstituted vesicles that contained only the HA and N glycoproteins of A/Victoria. Target cells incubated with SV served as a control. The results are shown in Fig. 6. There was no cross-reactivity between influenza A- and influenza B-specific effector cells. Specific lysis was demonstrated on the homologous targets and cross-reactive lysis to various degrees on the heterologous target cells. Effector cells that showed cross-reactivity for target cells infected with A/Victoria also produced significant lysis of target cells prepared with reconstituted vesicles that contained only the HA and N glycoproteins. Thus, antigen recognition by cross-reactive T cells is independent of the presence of M protein.

Lysis of Artificial Target Cells is H-2 Restricted. To confirm the observation that recognition of the artificial target cells is mediated by processes similar to those of targets prepared by infection the H-2 restriction of the cytolytic interaction was tested. Effector cells of the haplotypes H-2^b and H-2^k were tested on the appropriate target cells as well as on artificial cells prepared by fusion with A/Victoria vesicles. Table I shows that the H-2-restriction rule also applies to the target cells prepared with artificial vesicles.

Discussion

The aim of this study was to define the molecules that carry the specificities recognized by cross-reactive CTL that lyse target cells infected with serologically different influenza A subtypes. By using two types of target cells, both devoid of M protein, we found that cross-reactive T cells recognize antigens of the envelope

TABLE I
*H-2 Restriction of Cross-Reactivity Against Target Cells Prepared with A/
 Victoria Virus Vesicles*

Effector cell	Target			
	P-815-A/ Asia	P-815-A/ Vic-ves + SV*	L-929-A/ Asia	L-929-A/ Vic-ves + SV
	%			
DBA/2-A/Asia	68.5	32.6	3.2	4.8
CBA-A/Asia	4.3	5.6	45.4	22.3

Effector cells are derived from spleen cells of mice primed with A/Asia and restimulated in vitro for 6 d with A/Asia-infected syngeneic stimulator cells. The effector:target ratio was 25:1.

* Vic-ves, A/Victoria virus vesicles.

glycoproteins HA and N of influenza A viruses. This is in contrast to previous work that indicated a major role for M protein in providing the cross-reactive target antigen.

M protein, which is antigenically similar in all influenza A strains, was considered to be the candidate for the cross-reactive antigen seen by these T cells because recent studies have demonstrated that the M protein may be detected on the surface of infected cells (18-21). Braciale (8) has investigated the M protein specificity of CTL by interfering with the process of glycosylation. By showing that 2-deoxyglucose treatment of target cells had (a) no effect on M protein expression and (b) changed the competition pattern of cold target cells infected with homologous or heterologous influenza A subtypes, he concluded that the cross-reactive target antigen is a nonglycosylated protein and, therefore, perhaps, the M protein. However, the role of protein glycosylation for the target antigen recognition by CTL is unknown, and these conditions still allow HA synthesis of various degrees of glycosylation (32, 33). Furthermore, 2-deoxyglucose has profound inhibitory effects on T cell-mediated lysis (34).

We first investigated the role of M protein in determining the pattern of cross-reactivity in abortively infected cells. By abortive infection of L-929 cells with avian influenza A strains (FPV), target cells could be prepared that carry exclusively the HA and N proteins of influenza virus in their plasma membranes and are devoid of the M protein (23). These cells were used as targets for influenza A-specific CTL. When the two influenza-specific effector-cell subsets were tested on these target cells, the same pattern of cross-reactivity could be observed as had been found with target cells prepared with other influenza A strains. These results strongly argue against the role of M protein as a major antigen for cross-reactive T cell populations and indicate that these effector cells recognize antigens present on the HA and N glycoproteins. However, it remains possible that a low level of synthesis of M protein that we cannot detect occurs in these cells or that other components of the influenza virion may serve as target antigens.

With respect to the HA specificity of effector cells and the process of cross-reactivity, controversy exists with regard to the use of productively vs. abortively infected target cells (22, 35, 36). In some cases it has been suggested that incorporation of M protein into the plasma membrane of cells is even higher in abortively than in productively

infected cells (8, 20–22, 35–37). However, the opposite situation has been demonstrated in the model of the FPV infection of L-929 cells (23). Perhaps the major difference between the results obtained by different authors is a result of the different test conditions; for example, short- vs. long-term cytolytic assay and primary vs. secondary CTL.

Further evidence for the concept that the envelope glycoproteins carry the target antigens recognized by the cross-reactive T cell subset has been provided by target cells that carry the spike proteins of influenza virus exclusively and none of the influenza internal virion components. The preparation of those artificial target cells requires separation of the envelope proteins from the other viral components, reconstitution of the envelope proteins into lipid bilayers, and fusion of these artificial vesicles with the plasma membrane to generate target cells lysable by immune effector mechanisms (26). Preparation of pure envelope proteins from influenza virus strain A/Victoria was achieved after disruption of the virions in detergent followed by separation of the proteins on a sucrose gradient. For reconstitution of the detergent-solubilized proteins into lipid vesicles a mixture of four different phospholipids was used, and vesicle formation was achieved by a detergent-dilution procedure.

Reconstituted vesicles were smaller than virions. The packing of spikes in the lipid bilayer was found to be very compact by electron microscopy (EM). The ratio of HA:N spikes observed by EM was similar to that of the virus (38). These artificially reconstituted vesicles bind to the cell surface as revealed by immunofluorescence studies (data not shown). However, they do not fuse to any significant extent with the plasma membrane as cytolytic tests with antibody and complement (data not shown) and with cytolytic T cells were negative.

Vesicle-cell fusion can be achieved with polyethylene glycol (U. H. Koszinowski. Unpublished observations.). However, this method was poorly reproducible, and, thus, β -propiolactone-inactivated SV was used to promote fusion. This method offers some advantages. The fusion of SV with the cell membrane and its function in cell-cell fusion requires the presence of a functionally active fusion protein (16, 17). The fusion by SV can be monitored by the activity of SV-specific effector cells that are (a) selectively specific for SV and do not recognize influenza antigens and (b) also require the plasma membrane insertion of the SV antigens to lyse their targets. By using (a) two types of effector cells, one specific for SV and the other for influenza A antigens, and (b) target cells prepared either by infection with influenza A virus or by incubation with vesicles or mixtures of vesicles and fusion-active or fusion-inactive SV, the experimental system could be controlled for both antigenic specificities and for fusion. The molecular mechanism(s) responsible for fusion between the virus envelope and the plasma membrane and subsequent fusion between the plasma membranes and adjacent cells (or adjacent vesicles) in fusion from without is still unknown (39). In this study we have not tried to visualize the process of fusion itself. Therefore, the interpretation of the results as a result of fusion is based on the present knowledge about the conditions under which virus-induced cell fusion does occur (40).

Target cells prepared in this way were susceptible to the lysis of CTL specific for the homologous influenza A strain and for CTL with specificity for heterologous influenza A strains. The absence of M protein in the vesicles provided the formal proof that the cross-reactivity observed is not dependent upon the presence of the M

protein. Therefore, the nature of the antigens seen by this cell population must be solely a result of the antigens of the influenza HA and N glycoproteins. Although our preparations contained far more HA than N glycoproteins, we have not yet determined to which extent both envelope proteins contribute to the cross-reactive antigenic specificity. Work is in progress to prepare vesicles that contain either the HA or the N. Studies on the primary structure of the HA glycoprotein of different subtypes have made it possible to distinguish regions of variable and of conserved amino acid sequence. Thus it is quite possible for the HA to present a common structural determinant that could be recognized by cross-reactive T cells. It is also possible that the N, which is known to show much less antigenic variation, may carry a common determinant (41). Cross-reactivity of effector cells is also in accord with the observations of heterotypic immunity (10, 42, 43).

Recent data indicate that the specificity of CTL directed to influenza A virus antigens can be manipulated by different *in vitro* restimulation methods. With virions of heterologous strains as antigen for restimulation *in vitro*, the cross-reactive populations are predominantly triggered (4), whereas restimulation with homologous purified HA raises CTL with specificity for the viral HA (6, 44). If one assumes that in both cases determinants are recognized that are carried by the envelope proteins, the preparation of the antigens as either solubilized spikes or as proteins integrated and orientated in the cell membrane may decide which T cell population is triggered. The observation of cross-reactivity at the T cell level, which seems not to be reflected at the B cell level, is not without precedent (45, 46). Furthermore, the hypothetical M protein specificity of T cells also lacks an explanation because a primary influenza infection does not normally cause a significant antibody response to the M protein (2).

In assuming that the cross-reactive CTL are not less specific but recognize a common antigen shared by the glycoproteins, several explanations can be forwarded: (a) The number of B cell clonotypes (47, 48) to the cross-reactive antigens is normally too small to be detected. (b) The appropriate determinant cannot be selected by antigen-presenting cells for T cells that help B cells (49). (c) The B cell response to these antigens is normally suppressed (50). If the possibilities *b* and/or *c* do apply, one could predict that sensitization with inactivated or purified virions or viral protein preparations should give rise to similarly cross-reactive B cell clones. The preparation of defined molecules and their artificial integration in the plasma membrane may be a useful tool for the further analysis of T cell specificity.

Summary

Two populations of cytolytic T lymphocytes (CTL) generated after influenza A virus infection can be distinguished into one with specificity for the sensitizing hemagglutinin type and a second with cross-reactivity for antigens induced by other type-A influenza viruses. The molecules carrying the antigenic determinants recognized by the cross-reactive CTL were studied. In L-929 cells abortively infected with fowl plague virus, matrix (M) protein synthesis is specifically inhibited, whereas the envelope glycoproteins, hemagglutinin and neuraminidase, are synthesized and incorporated into the plasma membrane. These target cells were lysed by cross-reactive CTL.

The envelope proteins of type A/Victoria virus were separated from the other

virion components and reconstituted into lipid vesicles that lacked M protein that subsequently were used to prepare artificial target cells. Target-cell formation with vesicles was achieved by addition of fusion-active Sendai virus. These artificial target cells were also susceptible to lysis by cross-reactive CTL.

In contrast to previous observations that suggested that the M protein of influenza viruses is recognized by these effector cells, we present evidence that the antigenic determinants induced by the viral glycoproteins are recognized.

We thank Dr. R. Rott, of the University of Gießen (Gießen, Federal Republic of Germany), for providing the influenza strains and in particular for stimulating discussions. We are extremely grateful to Dr. N. Wrigley of the National Institute for Medical Research, London, for carrying out the electronmicroscopy. We are indebted to the Searle Laboratories (London) for the gift of purified A/Victoria virus. Ms. I. Neuschaefer-Rube provided skillful technical assistance, and the excellent secretarial help of Ms. U. Rohmann is gratefully acknowledged.

Received for publication 17 December 1979.

References

1. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* **27**:52.
2. Schild, G. C., and W. R. Dowdle. 1975. Influenza virus characterization and diagnostic serology. In *The Influenza Viruses and Influenza*. E. D. Kilbourne, editor. Academic Press, Inc., New York. 315.
3. Doherty, P. C., R. B. Effros, and J. Bennink. 1977. Heterogeneity of cytotoxic responses of thymus-derived lymphocytes after immunization with influenza virus. *Proc. Natl. Acad. Sci. U. S. A.* **74**:1209.
4. Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennink. 1977. Generation of both cross-reactive and virus-specific T cell populations after immunization with serologically distinct influenza A viruses. *J. Exp. Med.* **145**:557.
5. Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas. 1977. Cytotoxic T cells kill influenza virus-infected cells but do not distinguish between serologically distinct A viruses. *Nature (Lond.)* **267**:354.
6. Zweerink, J. 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.
7. Braciale, T. 1977. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus-strain specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell. Immunol.* **33**:423.
8. Braciale, T. 1977. Immunologic recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the cell surface and its role in recognition by cross-reactive cytotoxic T cells. *J. Exp. Med.* **146**:673.
9. Yap, K. L., and G. L. Ada. 1977. Cytotoxic T cells specific for influenza virus-infected target cells. *Immunology*. **32**:151.
10. Yap, K. L., and G. L. Ada. 1978. The recovery of mice from influenza A virus infection: adoptive transfer of immunity with influenza virus-specific cytotoxic T lymphocytes recognizing a common virion antigen. *Scand. J. Immunol.* **8**:413.
11. Effros, R. B., J. Bennink, and P. C. Doherty. 1978. Characteristics of secondary cytotoxic T cell responses in mice infected with influenza A viruses. *Cell. Immunol.* **36**:345.
12. Braciale, T., and K. L. Yap. 1978. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells. *J. Exp. Med.* **147**:1236.

13. Laver, W. G. 1973. The polypeptides of influenza viruses. *Adv. Virus Res.* **18**:57.
14. Choppin, P. W., and R. W. Compans. 1975. The structure of influenza virus. In *The Influenza Viruses and Influenza*. E. D. Kilbourne, editor. Academic Press, Inc., New York. 15.
15. Koszinowski, U., M.-J. Gething, and M. Waterfield. 1977. T cells cytotoxicity in the absence of viral protein synthesis in target cells. *Nature (Lond.)*. **267**:160.
16. Sugamura, K., K. Shimizu, D. A. Zarling, and F. H. Bach. 1977. Role of sendai virus fusion glycoprotein in target cell susceptibility to cytotoxic T cells. *Nature (Lond.)*. **270**:251.
17. 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.
18. Ada, G. L., and K. L. Yap. 1977. Matrix protein expressed at the surface of cells infected with influenza viruses. *Immunochemistry*. **14**:643.
19. Biddison, W. E., P. C. Doherty, and R. G. Webster. 1977. Antibody to influenza virus matrix protein detects a common antigen on the surface of cells infected with type A influenza viruses. *J. Exp. Med.* **146**:690.
20. Jackson, D. C., G. L. Ada, A. J. Hapel, and M. B. C. Dunlop. 1976. Changes in the surface of virus-infected cells recognized by cytotoxic T cells. *Scand. J. Immunol.* **5**:1021.
21. Ada, G. L., and K. L. Yap. 1979. The measurement of haemagglutinin and matrix protein present on the surface of influenza virus infected P-815 mastocytoma cells. *J. Gen. Virol.* **42**:541.
22. Ennis, F. A., W. J. Martin, and M. W. Verbonitz. 1977. Hemagglutinin-specific cytotoxic T-cell response during influenza infection. *J. Exp. Med.* **146**:893.
23. Lohmeyer, J., L. T. Talens, and H.-D. Klenk. 1979. Biosynthesis of the influenza virus envelope in abortive infection. *J. Gen. Virol.* **42**:73.
24. Helenius, A., E. Fries, and J. Kartenbeck. 1977. Reconstitution of Semliki forest virus membrane. *J. Cell Biol.* **75**:866.
25. Huang, R. T. C., K. Wahn, H.-D. Klenk, and R. Rott. 1979. Association of the envelope glycoproteins of influenza virus with liposomes—a model study on viral envelope assembly. *Virology*. **97**:212.
26. Morein, B., D. Barz, U. Koszinowski, and V. Schirmmacher. 1979. Integration of a virus membrane protein into the lipid bilayer of target cells as a prerequisite for immune cytolysis. *J. Exp. Med.* **150**:1383.
27. Critchley, D. R. 1974. Cell surface proteins of NIL 1 hamster fibroblasts labeled by a galactose oxidase, tritiated borohydride method. *Cell*. **3**:121.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature (Lond.)*. **227**:680.
29. Wang, K., and F. N. Richards. 1974. An approach to nearest neighbor analysis of membrane proteins. *J. Biol. Chem.* **249**:8004.
30. Koszinowski, U. H., and M. M. Simon. 1979. Generation of virus specific cytotoxic T cells in vitro. I. Induction conditions of primary and secondary virus specific cytotoxic T cells. *Eur. J. Immunol.* **9**:715.
31. Koszinowski, U. H., and M.-J. Gething. Generation of virus specific cytotoxic T cells in vitro. II. Induction requirements with functionally inactivated virus preparations. *Eur. J. Immunol.* In press.
32. Klenk, H.-D., R. T. Schwarz, M. F. G. Schmidt, and W. Woellert. 1978. The influenza virus hemagglutinin. *Top. Infect. Dis.* **3**:83.
33. Nakamura, K., and R. W. Compans. 1978. Effects of glucosamine, 2-deoxyglucose and tunicamycin on glycosylation, sulfation and assembly of influenza viral proteins. *Virology*. **84**:303.
34. MacDonald, H. R. 1977. Energy metabolism and T cell-mediated cytolysis. II. Selective inhibition of cytolysis by 2 deoxy-D-glucose. *J. Exp. Med.* **146**:710.
35. Ennis, F. A., W. J. Martin, M. W. Verbonitz, and G. M. Butchko. 1977. Specificity studies

- on cytotoxic thymus-derived lymphocytes reactive with influenza virus-infected cells: evidence for dual recognition of H-2 and viral hemagglutinin antigens. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3006.
36. Ennis, F. W., W. J. Martin, and M. W. Verbonitz. 1977. Cytotoxic T lymphocytes induced in mice by inactivated influenza virus vaccine. *Nature (Lond.)*. **269**:418.
 37. Gandhi, S. S., H. B. Bell, and D. C. Burke. 1971. Abortive infection of L cells by fowl plague virus: comparison of RNA and protein synthesis in infected chick and L cells. *J. Gen. Virol.* **13**:423.
 38. Wrigley, N. G. 1979. Electron microscopy of influenza virus. *Br. Med. Bull.* **35**:35.
 39. Poste, G., and C. A. Pasternak. 1978. Virus induced cell fusion. In *Cell Surface Reviews: Membrane Fusion*. G. Poste and G. L. Nicolson, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. **5**:305.
 40. Hosaka, Y., and K. Shimizu. 1977. Cell fusion by sendai virus. In *Cell Surface Reviews: Virus Infection and the Cell Surface*. G. Poste and G. L. Nicolson, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. **2**:129.
 41. Skehel, J. J., and M. D. Waterfield. 1975. Studies on the primary structure of the influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. U. S. A.* **72**:93.
 42. Kilbourne, E. D. 1978. Influenza as a problem in immunology. *J. Immunol.* **120**:1447.
 43. Floch, F., and G. H. Werner. 1978. Heterotypic protective immune reactions in mice infected with distinct serotypes of human influenza virus. *Ann. Microbiol. (Paris)*. **129**:509.
 44. Braciale, T. J. 1979. Specificity of cytotoxicity T cells directed to influenza virus hemagglutinin. *J. Exp. Med.* **149**:856.
 45. Hoffman, M., and J. W. Kappler. 1972. The antigen specificity of thymus derived helper T cells. *J. Immunol.* **108**:261.
 46. Kindred, B., and R. B. Corley. 1978. Specificity of helper T cells for different antigens. *Eur. J. Immunol.* **8**:67.
 47. Cancro, M. P., W. Gerhard, and N. R. Klinman. 1978. The diversity of the influenza-specific primary B-cell repertoire in BALB/c mice. *J. Exp. Med.* **147**:776.
 48. Yewdell, J. W., R. G. Webster, and W. U. Gerhard. 1979. Antigenic variation in three distinct determinants of an influenza type A hemagglutinin molecule. *Nature (Lond.)*. **279**:246.
 49. Rosenthal, A. S., M. A. Barcinski, and J. T. Blake. 1977. Macrophage selecting the appropriate determinant to be offered to different subpopulations of T cells. *Nature (Lond.)*. **267**:156.
 50. Yowell, R. L., B. A. Araneo, A. Miller, and E. E. Sercarz. 1979. Amputation of a suppressor determinant on lysozyme reveals underlying T cell reactivity to other determinants. *Nature (Lond.)*. **279**:70.