# IN VITRO GENERATION OF HUMAN
# CYTOTOXIC LYMPHOCYTES BY VIRUS

## Viral Glycoproteins Induce Nonspecific Cell-mediated
## Cytotoxicity without Release of Interferon*

By PAOLO CASALI, J. G. PATRICK SISSONS, MICHAEL J. BUCHMEIER, AND
MICHAEL B. A. OLDSTONE

*From the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla,
California 92037*

The outcome of a virus infection represents an imbalance toward either the infecting agent's virulence or the host's ability to limit virus spread. Limiting infection may be a function of the immune system and take the form of a specific response and/or result from a nonimmune (nonspecific) response. Recently, considerable attention has focused on nonspecific host responses because of their potential to halt or reduce viral replication early in infection before the generation of either specific antibodies or cytotoxic T lymphocytes. Examples of early antiviral mechanisms are serum complement that can lyse some virions (1, 2) and bind to infected cells (3-5) in the absence of antibodies, natural killer (NK)[1] cells (6) that lyse both infected and uninfected target cells (7, 8), and interferon, which can inhibit viral replication (9-12) as well as induce new or enhance ongoing NK cell activity (13-16).

The induction or enhancement of nonspecific cell-mediated cytotoxicity (CMC) (also called spontaneous CMC, natural CMC, or NK activity) by viruses and/or interferon has recently received much attention (6-8, 17-22). However, little is known about the role of viral polypeptides in induction or enhancement of this CMC. Early in infection, viral glycoprotein may be expressed on the surface of infected cells (23) before its release either into the fluid phase in soluble form or its incorporation into virions. For this reason, we studied the role of and mechanism(s) whereby soluble viral glycoproteins, viral glycoproteins inserted into artifical membranes (virosomes), and whole virions mediate nonspecific CMC.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; CMC, cell-mediated cytotoxicity; CTL, cytotoxic T lymphocytes; E, erythrocytes; EA, antibody-coated erythrocytes; EYPC, egg yolk phosphatidylcholine; FBS, fetal bovine serum; F, measles virus fusion glycoprotein; HA, measles virus hemagglutinin; K cells, killer cells; LCMV, lymphocytic choriomeningitis virus; LU, lytic units; MEM, minimum essential medium; NK, natural killer; NP-40, Nonidet P-40; octylglucoside, octyl-$\beta$-D-glycopyranoside; ORBC, ox erythrocytes; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; RDMC, rhabdomyosarcoma cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRBC, sheep erythrocytes; SSPE, subacute sclerosing panencephalitis; VSV, vescicular stomatitis virus.

We found that purified measles virus glycoproteins in a soluble form or inserted into phospholipid vesicles induced significant cytotoxic activity in peripheral blood lymphocytes (PBL) within 2–4 h of incubation. This CMC was expressed as lysis of autologous or heterologous cells and was not associated with the release of interferon in culture fluids. In contrast, whole virions required >8 h to generate the same degree of cytotoxic activity. This activity was associated with interferon release in the culture fluids. Glycoproteins purified from lymphocytic choriomeningitis virus (LCMV) duplicated this result, suggesting that these findings may be a generalized phenomenon.

## Materials and Methods

*Virus Source, Purification of Viral Polypeptides, and Their Integration in Artificial Lipid Membranes.* Measles virus and LCMV were used in this study. The source of measles virus, its cloning, passage history, method of propagation handling, and titration have been reported by this laboratory (3, 24, 25). To label virus, infected HeLa cells were incubated for 24 h in leucine-free Dulbecco's modified Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated dialyzed fetal bovine serum (FBS) (Flow Laboratories, Rockville, Md.), 1% glutamine, penicillin (100 IU/ml), streptomycin (100 $\mu$g/ml), and 6.25 $\mu$Ci/ml of [$^3$H]leucine (Amersham/Searle Corp., Arlington Heights, Ill.). Culture fluid containing measles virus was freed from debris by an initial centrifugation at 1,500 $g$ for 30 min at 4°C and the virus was then purified as described by this laboratory (24, 25). To prepare hemmagglutinin (HA) and fusion (F) glycoproteins, purified virus solubilized in 0.15 M NaCl, 0.01 M Tris-HCl buffer, (Tris-NaCl buffer), pH 8.0, containing 2% Nonidet P-40 (NP-40) (BDH Chemicals, Poole, England) was applied to a column of Sepharose beads bearing covalently linked *Lens culinaris* agglutinin (LCH Gel; E. Y. Laboratories Inc., San Mateo, Calif.) (25). After adsorption of applied material, the column was washed with Tris-NaCl buffer containing 0.1% NP-40, which was later replaced with Tris-NaCl buffer containing 30 mM octyl-$\beta$-D-glucopyranoside (octyl-glucoside; Calbiochem, La Jolla, Calif.). Solid-phase-bound measles virus glycoproteins were then eluted using Tris-NaCl buffer containing 30 mM octylglucoside and 0.1 M $\alpha$-methyl-mannoside. Protein concentration was determined by the Lowry method (26). As a control, a Sepharose-lentil-lectin column was run under similar conditions except that no solubilized virus was added. The fraction eluted from this column by $\alpha$-methyl-mannoside was found to be protein free. The source, cloning, preparation, and handling of LCMV have been described (27). LCMV glycoproteins were purified by affinity choromatography with two serial passages through a lentil-lectin column using 2% sodium cholate as a detergent. The purity of viral glycoproteins preparations were assayed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (25). Purified glycoproteins and the equivalent fraction eluted from the control column were freed of detergent by exhaustive dialysis at 4°C against phosphate-buffered saline (PBS). The integration of purified measles virus glycoproteins into lipid vesicles was carried out using egg yolk phosphatidylcholine (EYPC) (Sigma Chemical Co., St. Louis, Mo.) as reported (25). Traces of $^3$H-labeled measles virus glycoproteins and of $^{14}$C-labeled EYPC were used to check the integration of glycoproteins into the membranes by flotation gradient ultracentrifugation analysis (25).

*Immunochemical Reagents and SDS-PAGE.* Antibody to measles virus glycoproteins was obtained from the serum of a patient with subacute sclerosing panencephalitis (SSPE) and accounted for 10% of the total immunoglobulin (28). IgG antibodies and their F(ab')$_2$ fragments were prepared as reported (3). Serum from a healthy donor (SH) with no detectable anti-measles virus antibodies, as assayed by neutralization, fluorescent binding to virus-infected cells, and immune precipitation tests with $^{35}$S-labeled measles virus (24, 29), was used as a source of IgG or F(ab')$_2$ free of anti-measles viral antibodies. SDS-PAGE analysis of viral polypeptides was performed as described by Laemmli (30) and modified in this laboratory (24, 25).

*Cytotoxicity Studies.* PBL obtained from healthy measles immune and nonimmune subjects were used as effector cells in all cytotoxicity assays. Lymphocytes were obtained from heparin-

ized peripheral blood centrifugated on a Ficoll-Hypaque gradient (31) and freed of adherent cells by incubation for 1 h at 37°C with 5% $CO_2$ in a tissue culture flask (175 $cm^2$; Falcon Labware, Oxnard, Calif.). To obtain lymphocyte subsets, PBL were first incubated with neuraminidase- (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) treated sheep erythrocytes (SRBC) (32) and segregated into erythrocyte-rosette forming ($E^+$) cells and non-E-rosette forming ($E^-$) cells by application to a Ficoll-Hypaque gradient. Lymphocytes with ($C3^+$) and without ($C3^-$) receptors for C3 were separated by using a column of Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, N. J.) bearing activated human C3 molecules, as previously described (33). Lymphocytes bearing receptors for the Fc portion of IgG (Fc-positive cells) were removed from PBL by using a monolayer made of ox erythrocytes (ORBC) coated with rabbit anti-ORBC IgG. Tissue culture flasks (T-25; Falcon, Labware) previously treated with PBS containing 100 μg/ml of poly-L-lysine (70,000 mol wt) (Sigma Chemical Co.) were coated with ORBC during a 2-h incubation at room temperature. After washings, bound ORBC were allowed to react for 45 min at 37°C with an excess of rabbit anti-ORBC IgG. The erthrocyte antibody (EA) monolayer was then used to absorb Fc-positive cells by incubation for 1 h at 37°C. Under similar conditions, PBL were applied to an E monolayer (prepared as described for the EA monolayer, but without antibodies) to determine their degree of nonspecific binding.

Cell lines used as targets in this study were: adult human skin fibroblasts (for autologous cytotoxicity assays) and human fetal skin fibroblasts established in culture in our laboratory, K562 cells, rhabdomyosarcoma cells (RDMC; from the Wistar Institute) and Molt-4 cells. Fibroblasts and RDMC were cultured in MEM supplemented with 1% glutamine, 10% FBS, and antibiotics; K562 and Molt-4 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% glutamine, and antibiotics.

Cytotoxicity assays were carried out in 96-well plates (Costar, Cambridge, Mass.) using ^{51}Cr-labeled target cells as described (28) with slight modifications. Specific lysis was calculated by the following formula: where Spo is the ^{51}Cr release from targets in the presence of medium alone, Max is the ^{51}Cr release of target cells after treatment with 1% NP-40, and Sam is the ^{51}Cr release by the sample to be tested:

$$\text{percent specific release} = \frac{\text{Sam} - \text{Spo}}{\text{Max} - \text{Spo}} \times 100.$$

In most experiments, the effector to target cell ratio used was 100:1. When three effector to target cells ratios were used (100:1, 50:1, and 25:1), specific cytotoxicity was computed in lytic units (LU) over the number of effector cells by a modification of the Von Krogh equation (34). One LU is expressed as the number of lymphocytes required to lyse 50% of the target cells during the incubation time specified. All test cultures and controls were run in triplicate. Most often fibroblasts were subject to an 18-h assay; spontaneous release did not exceed 30%. Kinetic experiments with ^{51}Cr-labeled fibroblasts as targets were designed as follows: at time 0, four plates containing identical reagents were set up and incubated at 37°C in 5% $CO_2$. Each plate included samples for analysis of both spontaneous and maximal release from ^{51}Cr-labeled targets. At varying time-points, 200 μl of supernatant fluid was harvested from each well, 100 μl for determining ^{51}Cr release and 100 μl for titrating interferon-like activity.

*Interferon Assay.*  Interferon activity of supernatant fluids was tirated as their ability to inhibit the cytopathic effects of vesicular stomatitis virus (VSV) on a monolayer of Vero cells as described by Dahl (35) with minor modifications. All titrations were carried out in triplicate and interferon units were expressed as the reciprocal of the dilution inhibiting cytopathic effects by 50%. Each assay included Vero cells treated with a reference interferon preparation (National Institutes of Health Reference Interferon G-023-901-5271). Under the conditions used, one interferon unit derived in experiments was equivalent to one reference unit of NIH Human Reference Interferon.

## Results

*Soluble Measles Virus Glycoproteins Induced CMC.*  We first determined whether soluble measles virus glycoproteins and measles virions could induce PBL taken from several

human donors to kill heterologous and/or autologous fibroblast targets. Either 600 ng of purified measles virus glycoproteins, 3,000 ng of untreated or ultraviolet light (UV)-inactivated measles virions, or PBS was mixed with $10^6$, $5 \times 10^5$, or $2.5 \times 10^5$ PBL and $^{51}$Cr-labeled target cells, and the specific $^{51}$Cr release was determined over an 18-h period. Table I shows that PBL from all nine individuals studied mediated cytotoxicity. PBL incubated with untreated or UV-inactivated virions released significantly more $^{51}$Cr from both autologous and heterologous targets than did PBL incubated in PBS ($P = <0.02$). Soluble measles virus-glycoproteins induced as much or more killing by PBL as whole virions. One of the nine individuals, SH, had no history of measles virus infection and no antibody to measles, but her PBL were as effective as PBL from immune adults in inducing CMC after exposure to measles virus glycoproteins. Results were similar when PBL from these subjects were incubated with measles virus glycoproteins and tested against Molt-4, K-562, or RDMC target cells in a 4-h $^{51}$Cr release assay (data not shown). In contrast, incubation of these targets with virus glycoproteins in the absence of PBL did not yield any significant lysis. Additionally, the eluted fraction from a control Sepharose lentil-lectin column, devoid of measles virus glycoproteins, also failed to induce CMC.

We next determined the amounts of soluble measles virus glycoproteins required to cause PBL killing of target cells and the kinetics of lysis. Equivalent amounts of free glycoproteins and of glycoproteins associated with measles virions were studied for their induction of CMC. This was accomplished by using virions at protein concentration five times higher than that of soluble measles virus glycoproteins. Previous studies with lentil-lectin affinity chromatography indicated that glycoproteins accounted for ~20% of the whole virion protein (25). By establishing a dose-response curve, we noted a linear relationship between the amount of glycoproteins present and the percentage of specific $^{51}$Cr released from autologous fibroblasts in an 18-h assay (Fig. 1). As little as 100 ng of virus glycoproteins/$10^6$ PBL resulted in CMC.

TABLE I

Lysis of Heterologous and Autologous Fibroblasts by Human PBL in the Presence of Measles Virus Virions or Its Glycoproteins*

| Donor‡ | PBL incubated with | | | | | | | |
| | PBS only | | Virions | | | | Soluble virus glycoproteins | |
| | | | Untreated | | UV-inactivated | | | |
| | Heterologous | Autologous | Heterologous | Autologous | Heterologous | Autologous | Heterologous | Autologous |
|---|---|---|---|---|---|---|---|---|
| JH | 24 | 9 | 81 | 84 | 80 | 82 | 84 | 79 |
| MO | 16 | 34 | 79 | 73 | 75 | 70 | 138 | 131 |
| PS | 14 | 60 | 74 | 80 | 73 | 80 | 178 | 179 |
| FD | 57 | 15 | 95 | 141 | 90 | 135 | 191 | 211 |
| AB | 81 | — | 208 | — | 205 | — | 275 | — |
| BH | 20 | — | 171 | — | 180 | — | 238 | — |
| CB | 3 | — | 96 | — | 92 | — | 235 | — |
| LK | 3 | — | 142 | — | 109 | — | 138 | — |
| SH | 10 | — | 155 | — | 145 | — | 158 | — |
| | 25.9§ ± 26.2 | 30 ± 22.2 | 122.6 ± 48.2 | 95.0 ± 31.0 | 116.8 ± 48.8 | 91.9 ± 29.1 | 181.9 ± 59.9 | 150.2 ± 57.4 |

* An 18-h $^{51}$Cr release assay was used, and the data are presented as LU/$10^6$ PBL. The release of $^{51}$Cr from target cells alone over this timed period did not exceed 30%.

‡ SH was a donor with no clinical history of measles virus infection and no detectable circulating anti-measles virus antibodies. All other donors had detectable levels of anti-measles virus antibodies.
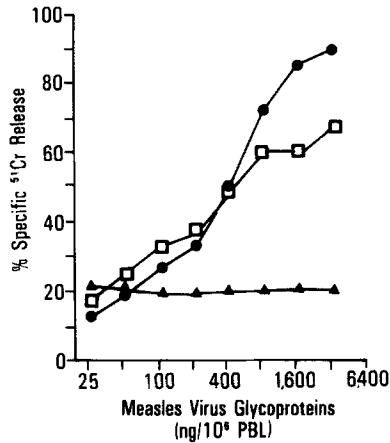
§ Mean value ± 1 SD.

FIG. 1.   Dose-response curve of CMC induced by measles virus. PBL were incubated with $^{51}$Cr-labeled autologous fibroblasts in the presence of increasing amounts of measles virus glycoproteins (●), whole virions (□), or PBS (▲). The effector to target cell ratio was 100:1 and the assay time was 18 h.

*Cell Binding Requirements of Measles Virus Glycoproteins for CMC.*   Next we determined whether the binding of virus glycoproteins to the surfaces of PBL and/or target fibroblasts was sufficient to induce CMC. After $1 \times 10^6$ PBL or $1 \times 10^4$ $^{51}$Cr-labeled autologous fibroblasts were preincubated with varying amounts of virus glycoproteins for 2 h at 37°C, the cells were either washed extensively to remove free glycoproteins or were transferred unwashed with their culture fluid (containing soluble glycoproteins) to plates containing untreated $^{51}$Cr fibroblasts and PBL, respectively. We found that PBL exposed to virus glycoproteins, washed, and then added to fibroblast targets induced CMC, but approximately six times more glycoproteins (2,900 ng/$10^6$ PBL) were needed to reach 50% lysis than when soluble viral glycoproteins were kept in the incubation fluids (500 ng/$10^6$ PBL) (Fig. 2 A). Similar results occurred when whole virions were used. CMC was induced when $^{51}$Cr-labeled fibroblasts were incubated with viral glycoproteins, washed, and added to untreated PBL. 10 times more glycoproteins (>10 μg/$10^4$ fibroblasts) were needed to achieve 50% of lysis than when the soluble glycoproteins were kept in the culture fluid (1.0 μg/$10^4$ fibroblasts) (Fig. 2 B). Radiolabeling studies showed that at 1.0 μg/$10^6$ PBL or $10^4$ fibroblasts, ~2% of offered measles virus glycoproteins became cell associated under the experimental conditions used. Hence, these studies indicated first that viral glycoproteins bound to fibroblasts could induce cytotoxic activity in unprimed PBL, and second, that as little as 10–20 ng viral glycoprotein bound to $10^6$ PBL induced significant CMC.

*Inhibition of Measles Virus Glycoprotein-induced CMC by Anti-Measles Virus Antibody.* F(ab')₂ fragments from an IgG fraction containing high titers of antibodies to measles virus and an IgG fraction devoid of anti-measles virus antibodies were compared for their ability to inhibit measles virus glycoprotein-induced CMC. PBS or PBS containing F(ab')₂ antibody in decreasing amounts from 4,500 to 4.4 μg were mixed a fixed amount of either measles virus glycoproteins (1.0 μg) or whole measles virus (5.0 μg) and incubated for 1 h at 37°C. Each mixture was then added to PBL (1 μg of glycoproteins/$10^6$ cells) and $^{51}$Cr-labeled autologous fibroblasts, and the specific $^{51}$Cr release was determined over 18 h (Fig. 3 A). F(ab')₂ antibody to measles virus (562
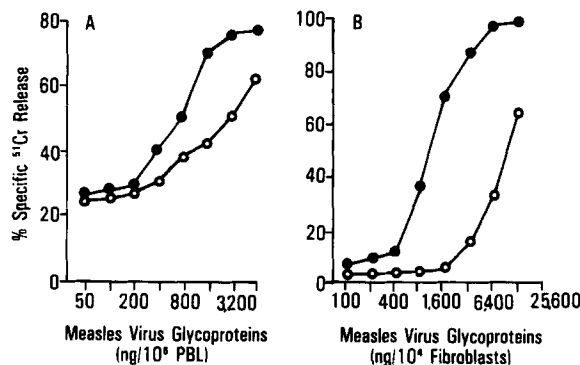
FIG. 2. Binding of measles virus glycoproteins to PBL or fibroblasts and enhancement of CMC. Panel A: resultant cytotoxicity against $^{51}$Cr-labeled autologous fibroblasts. PBL incubated with increasing amounts of viral glycoproteins were transferred with fluid-phase glycoproteins (●) or in the absence of soluble glycoproteins (○) into culture wells containing $^{51}$Cr-labeled heterologous fibroblasts. Panel B: $^{51}$Cr-labeled heterologous fibroblasts were incubated with increasing amounts of measles virus glycoproteins and then left in culture with glycoproteins (●) or glycoprotein containing medium was removed (○). Thereafter, untreated PBL were added. In both experiments, the effector to target cell ratio was 100:1 and the assay time was 18 h.
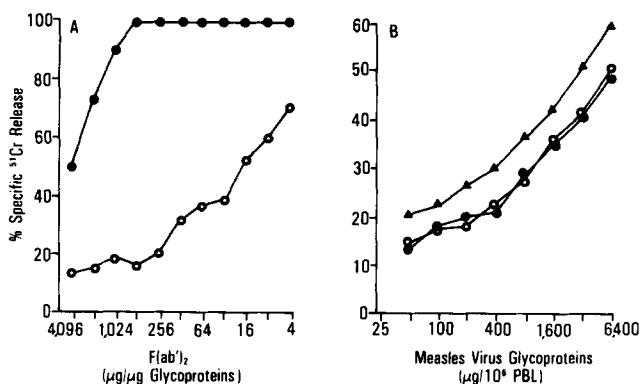


FIG. 3. Blocking of measles virus glycoprotein-induced CMC by anti-measles virus F(ab')₂. Panel A: measles virus glycoproteins were incubated with PBS containing decreasing amounts of F(ab')₂ free of antibodies to measles virus (●) or PBS containing F(ab')₂ with antibodies to measles virus (○) and then added to umprimed lymphocytes and $^{51}$Cr-labeled autologous fibroblasts. The effector to targer cell ratio was 50:1 and the assay time was 18 h. Panel B: incubation of PBL previously exposed to measles virus glycoproteins with anti-measles virus F(ab')₂. PBL were incubated for 2 h with increasing amounts of glycoproteins, washed, mixed with an excess of nonimmune (●) or immune F(ab')₂ (○) or PBS (▲) and added to $^{51}$Cr-labeled autologous fibroblasts. The effector to target cell ratio was 100:1 and the assay time was 18 h.

μg/μg glycoproteins) inhibited the cytotoxicity induced in PBL by both viral glycoprotein and whole virus by as much as 87 and 45%, respectively. In comparison, nonimmune F(ab')₂ was not effective (Fig. 3A).

Next, the ability of F(ab')₂ antiviral antibody to block the cytotoxicity of PBL that have been already exposed to virus glycoproteins was studied. Various amounts of glycoproteins (45–5,800 ng) were incubated with 10⁶ PBL for 2 h at 37°C. The PBL were washed extensively and added to either PBS, PBS containing F(ab')₂ antibody (600 μg/10⁶ PBL) to measles virus, or F(ab')₂ (600 μg/10⁶ PBL) preparation without antibody to measles virus. The mixtures were incubated with $^{51}$Cr-labeled autologous

fibroblasts and the amount of $^{51}$Cr released over 18 h was determined. As seen in Fig. 3 B, antibody to measles virus added to PBL previously exposed to virus glycoproteins did not abrogate their ability to lyse target cells.

*PBL Subsets Responsible for Viral Glycoproteins Induced CMC.* Positive and negative selection experiments to determine the effector PBL subsets involved in CMC were performed with PBL from two donors for each of the two separate experiments. Total PBL, E$^+$ cells, E$^-$ cells, E$^-$C3$^-$ cells, Fc$^+$ cells, and Fc$^-$ cells were assessed for the capacity to mediate CMC against $^{51}$Cr-labeled fibroblasts in the presence of PBS, PBS containing 900 ng of measles virus glycoproteins, or whole measles virions containing 4,500 ng of protein. As seen in Fig. 4, the Fc$^+$ PBL subset, not Fc$^-$ cells, exerted CMC. Assays of other markers in PBL subsets placed CMC activity both in E$^+$ and E$^-$ cells, but E$^-$ and C3$^-$ cells were the highest in activity. Hence, lymphocytes with and without E receptors mediate cytotoxicity induced by measles virus glycoproteins. The non-E-rosetting PBL mediating CMC are C3 receptor negative. However, all PBL mediating cytotoxicity induced by measles virus glycoproteins or by measles virus have Fc receptors.

*Segregation of CMC Induced by Soluble Virus Glycoproteins from That Induced by Virions.* Unexpected but significant differences in the time-course of CMC activity occurred when PBL were incubated with either soluble measles virus glycoprotein or untreated or UV-inactivated virions. As demonstrated in Fig. 5A and C, as soon as 4 h after adding 900 ng of soluble measles virus glycoproteins to 10$^6$ PBL, significant lysis of $^{51}$Cr-labeled autologous fibroblasts occurred. In marked contrast, equivalent lysis of fibroblasts by PBL incubated with untreated or UV-inactivated virions (4,500 ng of protein content) required 8 h or more of incubation. This difference in the early induction of CMC by soluble virus glycoprotein as compared with the later induction by whole virions was repeatedly observed in all experiments using PBL from eight individuals. Maximal $^{51}$Cr release occurring at 18 h was similar whether the PBL were incubated with soluble glycoproteins or virions.
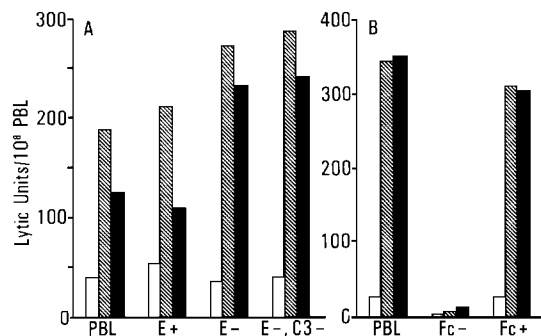


Fig. 4. Subsets of PBL responsible for CMC induced by viral glycoproteins. PBL or various subsets were assayed in the presence of PBS (▨), measles virus glycoproteins (☐), or whole measles virus (■). Panel A: positive selection experiment. PBL, nonadherent cells; E$^+$, lymphocytes forming rosettes with SRBC; E$^-$, lymphocytes not forming rosettes with SRBC; E$^-$C3$^-$, lymphocytes not forming rosettes with SRBC and without surface receptor for C3. Panel B: negative selection experiment. Fc$^+$ and Fc$^-$ PBL with or without surface receptor for the Fc fragment of IgG, respectively. In all experiments, the target cells were heterologous fibroblasts and the assay time was 18 h. The data represented were obtained by PBL from two donors. PBL from two other donors performed similarly.
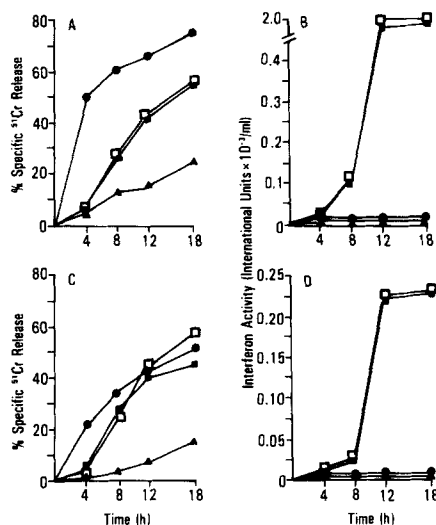
Fig. 5.   Differences between measles virus glycoproteins and measles virions in the induction of CMC and in interferon release. Panels A and C: killing of $^{51}$Cr-labeled fibroblasts by autologous PBL from two subjects; the effector to target cell ratio was 100:1. Panels B and D: the release of interferon into the fluid phase. PBL were exposed to PBS (▲), UV-inactivated measles virions (■), measles virions (□), or measles virus glycoproteins (●).

To further segregate these differences, we questioned whether soluble viral glyco-proteins or virions from measles virus induced interferon-like activity in the superna-tant fluids by PBL. In Fig. 5, panels B and D show that interferon was released into the culture medium only when untreated or UV-inactivated virions were present in culture with PBL. Interferon production peaked between 8 and 12 h, after which no further increase was demonstrable up to 18 h. The material was interferon-like in that it inhibited VSV replication, lost activity after trypsin treatment, and was not pelleted by ultracentrifugation (100,000 $g$/h). In none of our experiments did soluble measles virus glycoproteins incubated with PBL induce release of interferon-like activity in the culture medium. These findings were consistently reproduced and noted in PBL from several individuals.

We next determined whether the supernatant fluids harvested from the PBL-soluble glycoprotein cultures or from the PBL-virions cultures were able to induce CMC in newly added PBL. For this experiment, 1 × 10$^6$ PBL were incubated for 1 h at 37°C with either soluble measles virus glycoproteins (1,200 ng), virions (6,000 ng protein content), or PBS, and were washed and added to $^{51}$Cr-labeled or unlabeled autologous fibroblasts, or incubated without fibroblasts. After 6, 12, and 18 h of incubation, 150 µl of supernates from various cultures was UV-irradiated for 5 min and then added with untreated PBL and $^{51}$Cr-labeled fibroblasts in a 6-h culture. The degree of lysis achieved in the primary cultures is represented in Fig. 6A. Results shown in Fig. 6B indicate that media from PBL treated with whole virus and incubated with or without fibroblasts consistently induced CMC. In contrast, media from PBL preincubated with purified glycoproteins or PBS only, either in the presence or absence of fibroblasts, failed to induce cytotoxic activity in newly added PBL.

*Induction of CMC by Measles Virus Glycoproteins Integrated into Lipid Bilayers.*   We next determined whether these glycoproteins must be extracted from the viral envelope
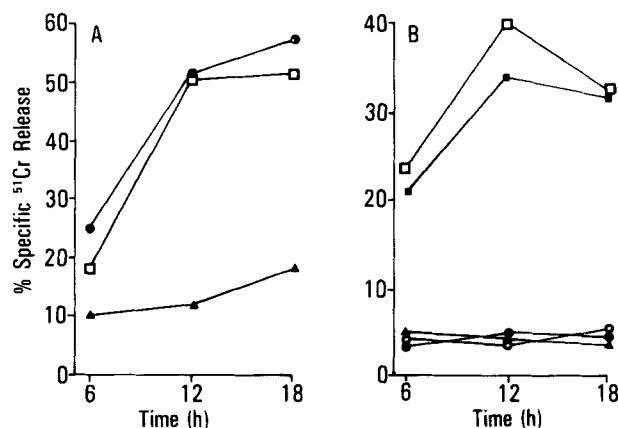
Fig. 6.   Recruitment of cytotoxic lymphocytes by fluids from cultures of PBL and measles virions or PBL and soluble glycoproteins. Panel A: CMC by PBL preincubated with measles virus glycoproteins (●), virions (□), or PBS (▲), washed and cultured with $^{51}$Cr-labeled heterologous fibroblasts for the indicated time. The effector to target cell ratio was 100:1. Panel B: CMC induction by culture fluids from PBL and measles virions with (■) or without (□) fibroblasts; culture fluids from PBL and glycoproteins with (●) or without (○) fibroblasts; culture fluids from PBL incubated with PBS in the presence of fibroblasts (▲). Culture fluids were harvested at indicated time. The effector to target cell ratio was 100:1 and the assay time was 6 h.

and presented in a free form to induce CMC, as in the foregoing experiments, or whether this activity is retained when the glycoproteins are integrated into a lipid bilayer. First, different amounts of purified measles virus glycoproteins from 28 ng to 3,600 ng, free or inserted into lipid bilayers, were added to $10^6$ PBL and cultured with 1 × $10^4$ $^{51}$Cr-labeled autologous fibroblasts. The amount of $^{51}$Cr released over 18 h was then determined. In a second study, $10^6$ PBL were incubated with 900 ng of purified measles virus glycoproteins, either in soluble form or integrated into vesicles, and $10^4$ $^{51}$Cr-labeled fibroblasts. The $^{51}$Cr release was measured after 4, 8, 12, and 18 h.

As shown in Fig. 7, measles virus glycoproteins inserted into artificial lipid membranes enhanced CMC to the same extent as an equivalent amount of their soluble counterparts (panel A), and with the same kinetics (panel B). Vesicles with integrated glycoprotein spikes (virosomes) did not induce release of mediators with interferon-like activity in the culture medium (data not shown). EYPC vesicles without the virus glycoproteins, "naked vesicles," did not induce any CMC against fibroblasts.

*Effect on CMC of Combining Measles Virus Glycoproteins and Virions.* To determine whether the virus glycoproteins and virions had an additive effect in inducing CMC, $10^6$ PBL were incubated with $10^4$ $^{51}$Cr-labeled fibroblasts and 1.2 µg of soluble measles virus glycoproteins, virions of measles virus containing 6 µg of protein, or both glycoproteins (1.2 µg) and virions (5 µg of protein). As seen in Fig. 8 A, by 12 h after incubation, the combined reagents (glycoprotein and virion) caused a lower CMC value than that resulting from incubation with measles virus glycoproteins alone. Results with UV-inactivated virions were similar to untreated virions. At 18 h, the combined effect of the two reagents was similar to that of glycoproteins or virus alone. The soluble measles virus glycoproteins did not interfere with the release of interferon
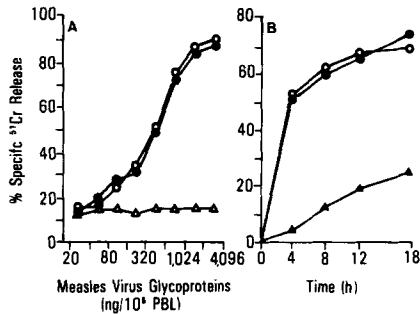
FIG. 7. CMC induced by measles virus glycoproteins integrated into artificial lipid membranes. Panel A: dose-response curve of CMC by PBL incubated with soluble measles virus glycoproteins (●), glycoproteins integrated into artificial membranes (O), and vescicles made with EYPC only (△). Panel B: kinetic study of CMC by PBL incubated with $^{51}$Cr-labeled autologous fibroblasts and soluble measles virus glycoproteins (●), glycoproteins integrated into artificial membranes (O), or PBS (▲). In both experiments, the effector to target cell ratio was 100:1 and the assay time was 18 h.
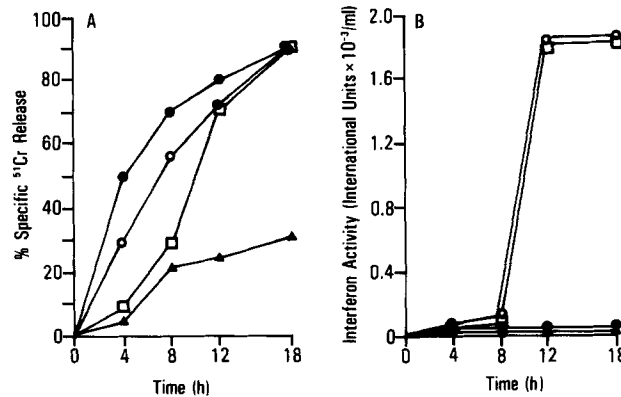


FIG. 8. CMC induced by measles virions or measles virus glycoproteins individually or both reagents combined together. Panel A: PBL incubated with $^{51}$Cr-labeled autologous fibroblasts in the presence of measles virus glycoproteins (●), a mixture of virions and glycoproteins (O), virions only (□), or PBS (▲). The effector to target cell ratio was 100:1 and the assay time 18 h. Panel B: the interferon activity of the respective culture media drawn at correspondent time-points to panel (A).

activity induced by the virions (Fig. 8B), because this activity was equal in culture fluids containing virions alone or with virions mixed with the glycoproteins.

*Soluble LCMV Glycoproteins Induced CMC.* PBL from several of the individuals tested above also killed human fibroblasts after incubation with purified LCMV glycoproteins and were evaluated by the same criteria used for measles virus. Purified LCMV glycoproteins enhanced CMC in a dose-response fashion as did measles virus glycoproteins (Fig. 1) and LCMV glycoprotein bound to the PBL as shown for measles virus glycoproteins (Fig. 2). In fact, LCMV soluble glycoproteins and whole virions behaved like the measles virus components in all parameters cited in this paper. Specifically, LCMV glycoproteins induced CMC faster than did whole LCM virions, and combining LCMV soluble glycoproteins and whole LCMV virions failed to produce a cumulative CMC effect. Additionally, only the virions, not purified glycoproteins, induced release of interferon by PBL. Further, purified LCMV nucleocapsids failed to induce CMC under similar experimental conditions.

## Discussion

Here we report a novel mechanism by which CMC can be initiated and expressed. Purified soluble measles virus HA and F glycoproteins alone or inserted into lipid bilayers induce or enhance human PBL killing of a variety of target cells including autologous and heterologous human fibroblasts, Molt-4, K-562, and RDMC cells. Studies of the kinetics of CMC induction, the absence of interferon in the culture fluids, and the inability of supernates to recruit cytotoxic activity from newly added PBL distinguish this type of nonspecific human CMC from the interferon-associated enhancement of NK cell activity (13, 17, 20). The segregation of various nonspecific CMC activities is outlined in Table II. Cells involved in these different effector mechanisms cannot yet be separated into discrete subsets according to conventional surface markers (Fig. 4).

The killing of target cells by PBL induced with soluble measles virus glycoproteins was efficient and occurred early. After first exposing PBL to glycoproteins and then transferring them to cultures of $^{51}$Cr-labeled targets, we found that as little as 10 ng of glycoprotein bound to PBL induced CMC. Further, kinetic studies showed that in most experiments, significant lysis (20–40% of total specific $^{51}$Cr release) occurred within 2–4 h of exposing target cells to PBL in the presence of viral glycoproteins. Virus glycoproteins inserted into lipid bilayers performed similarly, indicating that the activation of PBL was not dependent on the hydrophobic region of the glycoproteins. Previous electron microscopic and binding activity studies in this laboratory (25) have indicated that the hydrophobic parts of the measles virus glycoproteins are inserted into the vesicle's lipid bilayer and that the "spike projections" are arranged in patterns identical to those in virions. In contrast to this activity of free glycoproteins or glycoproteins inserted into lipid bilayers, measles virion-induced CMC in PBL takes three to four times longer to be expressed and kill comparable numbers and types of target cells. The dose-response curves for viral glycoproteins in soluble form, inserted into vescicles, or in the whole virion were equivalent in terms of CMC induction. However, the activity of soluble glycoproteins and virions combined was

TABLE II

*Subsets of Nonspecific CMC*

| CMC | | Lysis of targets at‡ | | | | Culture fluids | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Effector* | Inducer | 2 h | 4 h | 8 h | 18 h | Anti-viral activity§ | Capacity to induce new CMC |
| PBL | None (PBS) | Nil | Nil | ± | + | Nil | Nil |
| PBL | Soluble glycoproteins | + | ++ | +++ | ++++ | Nil | Nil |
| PBL | Glycoproteins inseted in lipid bilayer | + | ++ | +++ | ++++ | Nil | Nil |
| PBL | Virions | Nil | Nil | + | ++++ | ++++ | ++++ |

* Fc+ lymphocytes in both E− and E+ populations.
‡ This evaluation is based on data from multiple quantitative experiments carried out under conditions similar to those described in Fig. 6, using $^{51}$Cr-labeled autologous or heterologous fibroblasts as targets. Relative cytotoxic index with a value of 1.0 being maximum: ±, <0.1; +, 0.1–0.25; ++, 0.26–0.5; +++, 0.51–0.75; ++++, 0.76–1.0.
§ Interferon activity as judged by inhibition of cytopathic effect of VSV on Vero cells.

not additive but was intermediate between that of the two tested individually. This suggests that the whole virion per se might hamper early CMC induction due to glycoproteins alone. It is likely that both reagents compete for the same receptors on the cell's surface. The fact that UV-inactivated virions perform in a manner similar to their untreated counterparts suggests that replication of virus is not needed to inhibit early glycoprotein-induced CMC.

Supernatant fluids from PBL cultured in the presence of soluble viral glycoproteins or glycoproteins inserted in a lipid bilayer with or without fibroblasts did not contain interferon or other factors able to induce unprimed lymphocytes to be cytotoxic. In contrast, culture fluids from PBL incubated with virions, containing an equivalent amount of viral glycoproteins on their surface, displayed antiviral activity and were able to recruit lymphocytes to be cytotoxic.

Other experiments also indicate that viral glycoproteins absorbed on target cells are sufficient to induce CMC. Furthermore, the initiation of CMC activity by PBL with measles virus glycoproteins and virions was specific in that $F(ab')_2$ antibody to measles virus glycoprotein blocked the induction of CMC by first binding to the virus glycoproteins or virion. This prevented their binding to PBL. However, once the PBL had been exposed to glycoproteins, the antibody had no effect on inhibiting CMC activity, suggesting that glycoproteins, in part, directly activate lymphocytes to be cytotoxic. There are two recent studies concerning glycoproteins and activation of CMC in vitro. With PBL from humans, Härfast et al. (36) showed that mumps virus glycoproteins prepared by trypsinization of the virion induced CMC as did whole virions. With mouse PBL, an interferon-independent mechanism of activation of NK cells by alloantibodies with specificity for NK cells or by Helix pomatia lectin has been reported (37). Culture fluids from lymphocytes activated by these stimuli also failed to recruit new cytotoxic lymphocytes. Hence, the CMC induced in mouse lymphocytes by Helix pomatia lectin or alloantibodies to NK cells appears similar to that reported here in human lymphocytes by viral glycoproteins.

What could be the biologic implications of our findings? Cytotoxic lymphocytes play an important role in limiting virus infection and in surveillance against tumor cells, and by their lytic action also cause tissue injury that can harm the host. Experimental models showed that cytotoxic lymphocytes generated during a viral infection in vivo can be segregated into two main sets. One set is immunologically specific in terms of activation, recognition, and killing of target cells. Included within this set are specific cytotoxic T lymphocytes (CTL) and lymphocytes that kill through the recognition and binding of the Fc portion of specific anti-viral IgG (antibody-dependent cell-mediated cytotoxicity [ADCC]). In vivo studies indicate that specific CTL and IgG antibodies are not generated until 5–7 d after appropriate antigenic stimulation (38, 39). However, by this time the infecting virus can go through multiple cycles of replication (40).

The second set of CMC is generated earlier in vivo, usually within the first 24 h of infection (41), but lacks the fine target specificity shown by CTL and ADCC. NK cells are part of this second set of early, unspecific CMC. NK cells are generated in vitro within 6–12 h after exposure of PBL to virions, virus-infected, or tumor-bearing cells. NK cells segregate with the Fc receptor-bearing lymphocytes (42). In vivo and in vitro induction of NK activity is associated with the release of interferon by PBL. From the data in this paper, viral glycoprotein-induced CMC may constitute an

earlier line of defense, because its expression occurs already within 2-4 h after the exposure of lymphocytes to glycoproteins and interferon release is not required. Other experiments (P. Casali and M. B. A. Oldstone, manscript in preparation) indicate that similar to purified free glycoproteins, fibroblasts expressing measles virus HA and F polypeptides on their surfaces induce non-interferon-associated CMC within 2-4 h of incubation with PBL.

Thus, these early cellular mechanisms of defense against virus infection, comprising interferon-dependent as well as interferon-independent CMC, could help to limit virus spread by removing those cells that would otherwise later release infectious virus. Uninfected bystander cells could be killed in the microenvironment where these cells are operating. The combined action of killer cells induced by virus glycoproteins, virions, and interferon may function to limit both the spread of virus and the numbers of infected cells in primary infections until specific CTL and antiviral antibodies are generated much later. Failure of these early nonspecific defense mechanisms might favor the virulence of the virus over the host's immune system later in infection.

Our observatons with two dissimiliar RNA viruses, measles virus and LCMV, suggest that other viruses may perform similarly. Future work will determine both the generalities of these findings for other viruses and its biological significance in vivo.

## Summary

Purified hemagglutinin and fusion glycoproteins of measles virus either in soluble form or inserted in artifical membranes bind to human peripheral blood lymphocytes and induce cell-mediated cytotoxicity (CMC) in a dose-response fashion. Both autologous and heterologous noninfected target cells are lysed in vitro. The expression of CMC is not inhibited by anti-measles virus antibody added to lymphocytes previously exposed to viral glycoproteins. The killer lymphocytes are Fc receptor positive, both erythrocyte-rosetting and non-erthrocyte-rosetting, as assessed by both positive and negative selection experiments.

The induction of nonspecific CMC by viral glycoproteins either in the soluble state or inserted into artificial membranes could be segregated from the CMC associated with whole virions. First, on kinetics studies, purified viral glycoproteins induced CMC more rapidly than did whole virions. Second, viral glycoprotein-produced response occurred in the absence of detectable release of interferon into the culture medium, whereas CMC activity due to whole virions was associated with interferon release. The fact that purified measles virus glycoproteins integrated into artificial membrane bilayers were as efficient as their soluble counterparts in inducing CMC suggests that the hydrophobic portion of the glycoproteins was not involved in the induction and expression of the lytic activity. Purified glycoproteins from lymphocytic choriomeningitis virus behave similarly, although this virus is unrelated to measles virus. It is inferred that interferon-independent CMC induced by viral glycoproteins might account for some of the biological reactions occurring early in the control of a viral infection.

# References

1. Oldstone, M. B. A. 1975. Virus neutralization and virus-induced immune-complexes disease: virus-antibody union resulting in immunoprotection or immunologic injury—two different sides of the same coin. *Prog. Med. Virol.* **10:**84.

2. Cooper, N. R., and M. R. Welsh. 1979. Antibody and complement-dependent viral neutralization. *Springer Sem. Immunopathol.* **2:**285.

3. Perrin, L. H., B. S. Joseph, N. R. Cooper, and M. B. A. Oldsteon. 1976. Mechanism of injury of virus-infected cells by antiviral antibody and complement: participation of IgG, F(ab')₂ and the alternative complement pathway. *J. Exp. Med.* **143:**1027.

4. Sissons, J. G. P., M. B. A. Oldstone, and R. D. Schreiber. 1980. Antibody independent activation of the alternative complement pathway by measles virus infected cells. *Proc. Natl. Acad. Sci. U. S. A.* **77:**559.

5. Sissons, J. G. P., and M. B. A. Oldstone. 1980. Antibody-mediated destruction of virus infected cells. *Adv. Immunol.* **29:**209.

6. Herberman, R. B. 1980. *In* Natural Cell-mediated Immunity against Tumors. Academic Press, Inc., New York. 1.

7. Santoli, D., and H. Koprowski. 1979. Mechanisms of activation of human natural killer cells against tumor and virus infected cells. *Immunol. Rev.* **44:**125.

8. Herberman, R. B., J. Y. Djeu, D. H. Kay, J. R. Ortaldo, C. Riccardi, G. D. Bonnard, H. T. Holden, R. Fagnani, A. Santoni, and P. Puccetti. 1979. Natural killer cells: characteristic and regulation of activity. *Immunol. Rev.* **44:**43.

9. Notkins, A. L. 1975. Interferon as a mediator of cellular immunity in viral infections. *In* Viral Immunology and Immunopathology. A. L. Notkins, editor. Academic Press, Inc., New York. 149.

10. Gresser, I., M. G. Tovey, M. T. Bandu, C. Maury, and D. Brouty-Bogye. 1976. Role of interferon in the pathogenesis of virus disease in mice as demonstrated by the use of an anti-interferon serum. I. Rapid evolution of encephalomycarditis virus infection. *J. Exp. Med.* **144:**1305.

11. Stewart, W. E. 1979. Mechanisms of antiviral actions of interferons. *In* The Interferon System. Springer-Verlag, New York. 196.

12. Gresser, I. 1977. On the varied biological effects of interferon. *Cell. Immunol.* **34:**406.

13. Trinchieri, G., and D. Santoli. 1978. Antiviral activity induced by culturing lymphocytes with tumor-derived or virus transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J. Exp. Med.* **147:**1314.

14. Gidlund, D., A. Örn, H. Wigzell, A. Senik, and I. Gresser. 1978. Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature (Lond.).* **273:**759.

15. Minato, N., B. R. Bloom, C. Jones, J. Holland, and L. M. Reid. 1979. Mechanism of rejection of virus persistently infected tumor cells by athymic nude mice. *J. Exp. Med.* **149:**1117.

16. Djeu, J. Y., K. Y. Huang, and R. B. Herberman. 1980. Augmentation of mouse natural killer activity and induction of interferon by tumor cells in vivo. *J. Exp. Med.* **151:**781.

17. Trinchieri, G., B. Perussia, and D. Santoli. 1980. Spontaneous cell-mediated cytotoxicity modulation by interferon. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 655.

18. Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. A. Oldstone. 1981. The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Adv. Immunol.* **30**:274.

19. Bloom, B., N. Minato, A. Neighbour, L. Reid, and D. Marcus. 1980. Interferon and NK cells in resistance to persistently virus-infected cells and tumors. *In* Natural Cell-Mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 505.

20. Bloom, B. 1980. Interferons and the immune system. *Nature (Lond.)*. **284**:593.

21. Saksela, E., T. Timonen, I. Virtaneu, and K. Cantell. 1980. Regulation of human natural killer activity by interferon. *In* Natural Cell-mediated immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 645.

22. Brunda, J. M., H. T. Holden, and R. B. Herberman. 1980. Augmentation of natural killer cell activity of beige mice by interferon and interferon inducers. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 411.

23. Oldstone, M. B. A., R. S. Fujinami, and P. W. Lampert. 1980. Membrane and cytoplasmic changes in virus-infected cells induced by interactions of antiviral antibody with surface antigens. *Prog. Med. Virol.* **26**:45.

24. Fujinami, R. S., and M. B. A. Oldstone. 1980. Alteration in expression of measles virus polypeptides by antibody: molecular events in antibody-induced antigenic modulation. *J. Immunol.* **125**:78.

25. Casali, P., J. G. P. Sissons, R. S. Fujinami, and M. B. A. Oldstone. 1981. Purification of measles virus glycoproteins and their integration into artificial lipid membranes. *J. Gen. Virol.* **54**:161.

26. 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.

27. Buchmeier, M. J., J. H. Elder, and M. B. A. Oldstone. 1978. Protein structure of lymphocytic choriomeningitis virus: identification of the virus structural and cell associated polypeptides. *Virology.* **89**:133.

28. Perrin, L. H., A. Tishon, and M. B. A. Oldstone. 1977. Immunologic injury in measles virus infection. III. Presence and characterization of human cytotoxic lymphocytes. *J. Immunol.* **118**:282.

29. Sissons, J. G. P., N. R. Cooper, and M. B. A. Oldstone. 1979. Alternative complement pathway-mediated lysis of measles virus infected cells: induction by IgG antibody bound to individual viral glycoproteins and comparative efficacy of F(ab′)₂ and Fab′ fragments. *J. Immunol.* **123**:2144.

30. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680.

31. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand J. Clin. Lab. Invest.* **21**(suppl. 97):1.

32. Wahl, S. M., D. L. Rosenstreich, and J. J. Oppenheim. 1976. Separation of human lymphocytes by E rosettes sedimentation. *In* In Vitro Methods in Cell-mediated and Tumor Immunity. B. R. Bloom and J. R. David, editors. Academic Press, Inc., New York. 241.

33. Casali, P., and B. Perussia. 1977. C3-reacted sepharose: a preparative method for separating T and B lymphocytes. *Clin. Exp. Immunol.* **27**:38.

34. Trinchieri, G., M. De Marchi, W. Mayr, M Savi, and R. Cepellini. 1973. Lymphocyte antibody lymphocytolytic interaction (LALI) with special emphasis on HLA. *Transpl. Proc.* **5**:1631.

35. Dahl, H. 1973. A micro assay for mouse and human interferon. II. Dose-response in different cell-virus systems. *Acta Pathol. Microbiol. Scand.* **B18**:359.

36. Härfast, B., C. Örvell, A. Alsheikhly, T. Anderson, P. Perlmann, and E. Norrby. 1980. The role of viral glycoproteins in mumps-virus dependent lymphocyte-mediated cytotoxicity *in vitro. Scand. J. Immunol.* **11**:391.

37. Brunda, M. J., R. B. Herberman, and H. T. Holden. 1980. Interferon-independent

activation of murine natural killer cells activity. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 525.

38. Perrin, L. H., R. M. Zinkernagel, and M. B. A. Oldstone. 1977. Immune response in humans after vaccination with vacuria virus: generation of a virus-specific cytotoxic activity by human peripheral lymphocytes. *J. Exp. Med.* **146:**949.

39. 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:**51.

40. Joklik, W. K. 1980. The virus multiplication cycle. *In* Principles of Animal Virology. W. K. Joklik, editor. Appleton-Century Crofts, New York. 76.

41. Welsh, R. M. 1981. Natural killer cells in virus infection. *In* Current Topics in Microbiology and Immunology. O. Haller, editor. Springer-Verlag, Berlin. In press.

42. Sakasala, E., T. Timonen, A. Ranki, and P. Häjry. 1980. Morphological and functional characterization of isolated effector cells responsible for human natural killer cell activity to fetal fibroblasts and to cultured cell line targets. *Immunol. Rev.* **44:**71.