Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2456–2459, April 1985
Immunology

Natural killer cell recognition of target cells expressing different antigens of vesicular stomatitis virus

(viral immunity/immune surveillance/gene transfer/ts mutants and cell surface antigens)

JOHANNA R. MOLLER*, BRACHA RAGER-ZISMAN*, PHUC-CAHN QUAN*, AMICHAI SCHATTNER*, DAVID PANUSH*, JOHN K. ROSE†, AND BARRY R. BLOOM*‡

Departments of *Microbiology and Immunology and of ‡Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461; and †The Salk Institute, Department of Molecular Biology and Virology, La Jolla, CA 92138

Communicated by Matthew D. Scharff, December 10, 1984

ABSTRACT **Natural killer (NK) cells have the capability of lysing virus-infected, transformed, and embryonal cells, yet the nature of the target structure(s) recognized remains unclear. The availability of well-characterized temperature-sensitive (ts) mutants of vesicular stomatitis virus, defective in expression of individual viral-encoded polypeptides at the nonpermissive temperature (39°C), offered an approach to elucidating NK-cell recognition of virus-infected cells. Target cells were infected with ts mutants in three functions: the viral surface glycoprotein (G protein; ts 045); the matrix (M) protein (ts G31, ts G33), and the polymerase (ts G11). Cells infected with wild-type virus and all ts mutants at the permissive temperature (31°C) were killed by murine spleen cells. Similar to results on cytotoxic T lymphocytes, target cells infected by ts 045 defective in expression of G protein at 39°C were not killed by NK cells. Unexpectedly, cells infected at 39°C with the M-protein mutants also were not killed, although G protein was expressed at the cell surface. Target binding studies indicated that conjugates were not formed by cells infected with the ts mutants at the nonpermissive temperature. That expression of G protein was not sufficient for NK cell-mediated cytotoxicity was established in experiments in which a plasmid (pSVGL) containing the gene for vesicular stomatitis virus G protein was transfected into COS cells. Although G antigen was expressed on the plasma membrane, the cells were not lysed. These results suggest either that recognition of virus-infected cells depends on an appropriate conformation imparted to the viral G protein by association with the M protein or that NK cells can recognize alterations in the structure of the cell membrane induced by insertion of viral M and G molecules.**

Natural killer (NK) cells have the ability to discriminate between virus-infected and uninfected target cells and between some tumor and normal cells. NK-cell recognition of target cells appears to be nonspecific in the immunological sense and unrestricted in major histocompatibility (1–3) yet is highly "selective" for certain target structures. The nature of these structures remains problematic.

Early it was suggested that retrovirus-encoded antigens were the major target structures for NK cells (4), but a number of exceptions in murine systems rapidly materialized (5). High molecular weight viral glycoproteins (G proteins) that inhibit killing have been isolated from target cells (6), but they have not been further characterized or shown to represent target structures. In studies on NK-susceptible lymphoma cells and NK-resistant variants derived from them, the ganglioside asialo-GM_2 has been associated with NK-cell recognition (7), although several NK susceptible target cells lack this ganglioside.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Because of the availability of well-characterized conditional-lethal mutants of vesicular stomatitis virus (VSV) (8), which were useful in characterizing recognition by cytotoxic T lymphocytes (CTL) (9), and of techniques for gene transfer of certain VSV genes (10), we attempted to explore the role of viral-encoded polypeptides and glycoproteins in recognition of target cells by NK cells.

MATERIALS AND METHODS

Viruses. The Indiana (HRC) serotype of VSV was used and grown in primary chicken embryo fibroblasts. Wild-type (wt) VSV and the mutants ts (temperature sensitive) G31 defective in the matrix (M) protein and ts G11 defective in viral polymerase (L protein) were kindly provided by P. I. Marcus. Mutants ts 045 defective in G protein and ts G33 defective in M protein were kindly provided by H. Lodish. All VSV ts mutants were propagated on Vero cell monolayers at the permissive temperature of 31°C (10).

Cells. An NK-resistant subclone was selected from clone M2R derived from B16 mouse melanoma cells (11) and propagated in Dulbecco's modified Eagle's (DME) medium/F-12 medium supplemented with 10% fetal calf serum and antibiotics. COS cells were grown in DME medium supplemented as above. P815 mastocytoma cells were maintained in RPMI 1640 medium with the same supplements.

Antisera. Polyclonal rabbit anti-VSV, anti-VSV G protein, and anti-VSV M protein antisera were generously provided by J. Holland. Monoclonal anti-VSV G ascites was a gift from R. Weiss, H. Koprowski, and M. Pasternak. Sheep anti-mouse interferon (IFN) globulin (12) was kindly provided by I. Gresser. At a dilution of 1:1000, it completely neutralized 200 units of mouse IFN-α.

Transfection of the Gene for VSV G Protein into COS Cells. The plasmid pSVGL, containing the gene for the VSV G protein, was prepared as described (13), and 30 μg was added to 10^6 COS cells in the presence of 500 μg of DEAE-dextran per ml in serum-free medium. After 3 hr, medium was replaced and the cells were incubated further for 40–45 hr. The COS cells were stained with monoclonal antibody to VSV G protein for 30 min, washed, stained with a rabbit anti-mouse fluorescein isothiocyanate-conjugated globulin, washed, and diluted to 0.5–1 $\times$ 10^6 cells per ml. Approximately 20–30% of the cells expressed surface G protein. To enrich for antigen-expressing cells, the transfected cells were sorted by the fluorescence-activated cell sorter (FACS), and the percentage of surface G antigen-positive cells was increased to 80%.

Abbreviations: VSV, vesicular stomatitis virus; NK, natural killer; CTL, cytotoxic T lymphocytes; TBC, target-binding cells; moi, multiplicity of infection; IFN, interferon; FACS, fluorescence-activated cell sorter; ts, temperature sensitive; wt, wild type; G protein, viral glycoprotein; M protein, viral matrix protein.

RESULTS

Antigen Expression and NK Cell Lysis of Target Cells Infected with VSV ts Mutants at Permissive (31°C) and Nonpermissive (39°C) Temperatures. wt VSV replicates equally well at 31°C and at 39°C. In contrast, viral synthesis and assembly of ts mutants of VSV proceed normally at 31°C, but little infectious virus is produced by the ts mutants at 39°C. Mutant ts 045 is defective in production of the G protein normally displayed on the outer surface of the plasma membrane, and surface immunofluorescence was not seen at the nonpermissive temperature (39°C). The ts mutants G31 and G33 are defective in production of M protein, which is associated with the cytoplasmic nucleocapsids and serves as a matrix for the association of the nucleocapsid with the cytoplasmic domain of the transmembrane G protein. Of interest is the finding that at nonpermissive conditions, where M protein was not detectable in the cytoplasm of cells infected by the M-protein mutants, G protein was invariably found by staining with monoclonal or polyclonal anti-G protein antibodies on the cell surface.

NK cell cytotoxicity of two virus-infected targets was comparable for target cells infected at 31°C or 39°C by wt VSV (Table 1). That viral replication is required for lysis was indicated by the finding that cells infected with UV-inactivated VSV were not lysed, even though virus could be adsorbed to the surface. Cells infected with all of the ts mutants showed killing by NK cells at 31°C, and targets infected with ts G31 were consistently found to have significantly higher degrees of lysis. It is well known that the mutation in G31 causes an overaccumulation of several viral proteins (8). The phenotypes of the effector cells in this system were predominantly $NK1.1^+$ and $Thy\text{-}1.2^+$ (data not shown), corresponding to the NK_I and NK_T subsets previously described (15).

When cells infected at the nonpermissive temperature were tested for susceptibility to NK cells, killing of the G protein mutant (ts 045) was not observed. These results indicated, as in previous studies on CTL (9), that expression of the G protein was important for NK recognition. In contrast to results with CTL, target cells infected with M protein mutants, G31 and G33, were similarly not lysed at 39°C. (The low level of lysis of the COS cells was due to M-antigen expression during the 8 hr of NK cell assay at 37°C.) This was particularly unexpected because surface G protein could be detected in M protein mutant-infected cells at 39°C by immunofluorescence or FACS analysis (Fig. 1). Finally, as expected, cells infected with mutant ts G11, defective in the viral polymerase and unable to synthesize viral mRNA or proteins at nonpermissive temperature, were not killed.

Table 1. NK cell lysis of cells infected with VSV ts mutants at permissive (31°C) and nonpermissive (39°C) temperatures

Virus	Affected protein	% specific lysis			
		B16 cells		COS cells	
		31°C	39°C	31°C	39°C
None	—	6 ± 4	7 ± 3	4 ± 4	8 ± 3
UV-inactivated VSV	—	0	0	NT	NT
VSV (wt)	—	23 ± 4	22 ± 5	NT	NT
ts 045	G	20 ± 3	1 ± 3	27 ± 6	12 ± 4
ts G31	M	28 ± 8	6 ± 4	48 ± 4	20 ± 3
ts G33	M	32 ± 3	0	38 ± 5	11 ± 2
ts G11	L	15 ± 4	2 ± 2	NT	NT

B16 melanoma cells were infected and incubated for 16 hr with wt VSV or ts mutants of VSV at a multiplicity of infection (moi) of 0.001–0.1. COS cells were infected for 1.5 hr with ts mutants at a moi of 10, washed, and incubated for 5 hr at 31°C or 39°C. The ^{51}Cr-release assays were then performed at 37°C for 8–10 hr at a lymphocyte-to-target ratio of 100:1 as described (14). The results are the average of triplicates of four experiments (± SEM); spontaneous release did not exceed 30%. The 20% specific lysis of ts G-31 at 39°C is explained by the leakiness of the virus (confirmed by antigen expression) during the ^{51}Cr-release assay at 37°C for 8–10 hr. L protein, viral polymerase; NT, not tested.

Inability of Anti-VSV Antiserum or Anti-interferon (IFN) Antiserum to Inhibit Lysis of VSV-Infected Cells. The presence of specific anti-VSV antibodies (Table 2) sufficient to neutralize 10^5 plaque-forming units, 100-fold more virus than ever detected in these cultures, failed to block NK cell killing of VSV-infected cells as in other viral systems (16). In addition, monoclonal antibodies to VSV G or M protein had no effect on lysis of VSV-infected B16 cells or COS cells. Similar results were obtained when sheep anti-IFN antiserum was added to the effector cell/target cell mixtures, as reported previously (15), indicating that any IFN produced in the assay did not affect the lysis observed in this system.

Lymphocyte Binding to Target Cells Infected with VSV ts Mutants. The elegant methods developed (17) for analysis of the interaction of lymphocytes and targets at the single-cell level allow the discrimination between NK cell recognition of target cells (target cell binding) and lytic events. Because the assay has been developed only for nonadherent targets and preliminary studies on monolayers of infected B16 melanoma cells were unsatisfactory, we applied the assay to nonadherent P815 cells, which bind NK cells poorly and are resistant to lysis (Table 3). P815 cells infected with wt VSV at 31°C formed approximately 20% target-binding cells (TBC). Similar numbers of TBC were observed when P815 was infected at the permissive temperature with M-protein (ts G31) and G-protein (ts 045) mutants. However, a significant reduction in TBC was observed when cells were infected with either mutant at the nonpermissive temperature (39°C). Thus, the recognition of target structures in VSV-infected cells requires expression of both G and M proteins.

It could be argued that, since most cells have receptors for VSV, the target cell binding observed was solely due to binding of lymphocytes through receptors for VSV. Two lines of evidence render this possibility unlikely. When EL4, a continuous T-cell line, was added to VSV-infected and uninfected P815 cells, there was no difference in binding (9 conjugates per 200 uninfected cells vs. 10 conjugates per 229 infected cells). Secondly, binding of splenic lymphocytes was not inhibited by hyperimmune rabbit anti-VSV antiserum.

NK Cell Cytotoxicity of COS Cells Transfected with the Plasmid pSVGL Encoding the VSV G Protein. Transfection of COS cells with a plasmid construct, pSVGL, containing the intact gene for the VSV G protein results in the expression and membrane association of G protein in these cells for a period up to 48–60 hr (13, 18). Since this plasmid expresses G protein most efficiently in COS cells, we used these cells to test rigorously whether expression of the G protein on the membrane of target cells was a sufficient condition for recognition and killing by NK cells. COS cells were transfected with pSVGL and tested for susceptibility to killing by spleen cells of nude mice either directly or after enrichment for COS cells expressing G protein by cell sorting. By FACS analysis 20% of the cells transfected with pSVGL expressed surface VSV G protein (above background), and after cell sorting 77% of the recovered cells expressed VSV G protein. Comparable figures were seen in COS cells infected with VSV ts G31 or G33. While cytotoxicity of the cells infected with VSV ts G31 at 37°C was obtained (Table 4), the cells transfected with pSVGL and expressing surface G protein were totally refractory to killing by NK cells. Since the transfection experiments indicated that expression of G protein was insufficient for rendering COS cells susceptible to NK killing, we explored the influence of M protein expression on killing of COS cells. As shown in Fig. 1, FACS analysis of COS cells, incubated for 5 hr after infection with the M protein mutants, expressed equal amounts of G protein at

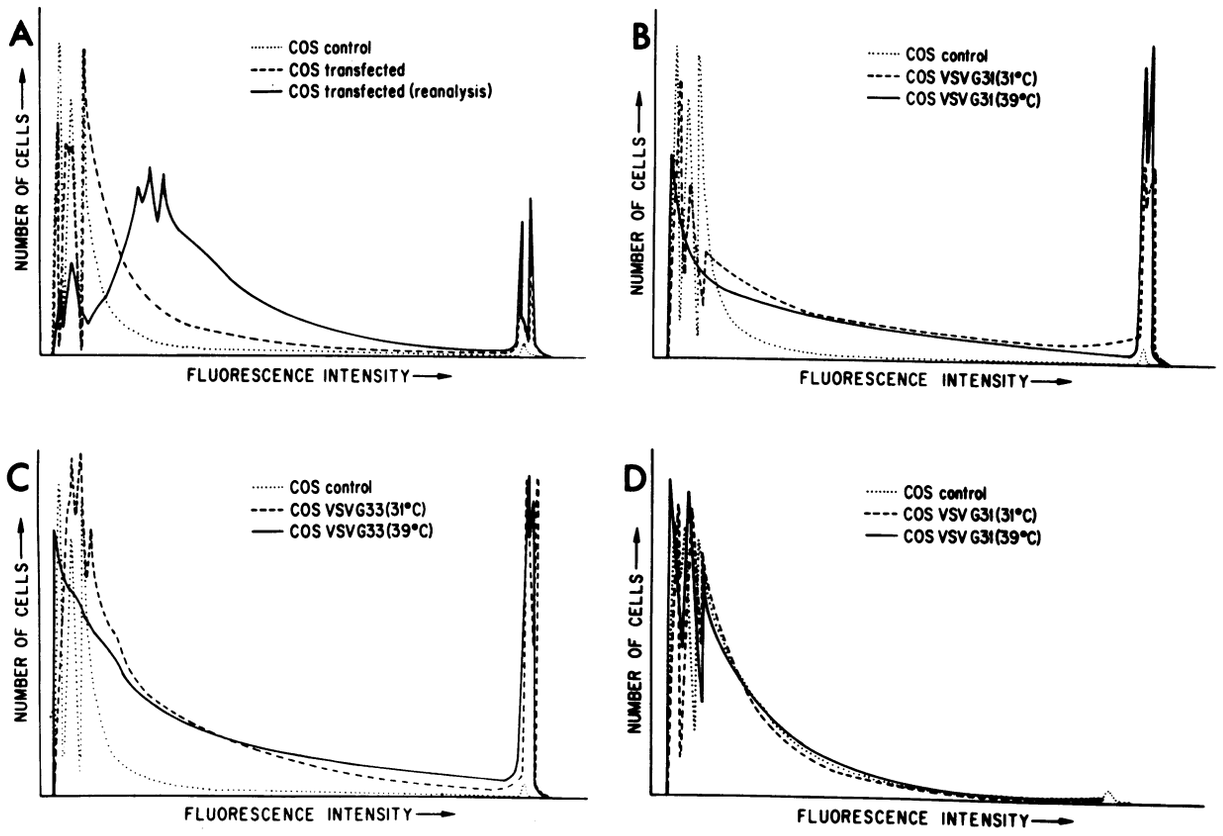

FIG. 1. FACS analysis of COS cells stained with monoclonal anti-VSV G protein antiserum (10% of the COS control cells are positive. (*A*) COS cells transfected with the plasmid pSVGL before (30% positive) and after (80% positive) cell sorting. (*B*) COS cells infected with VSV G31 for 5 hr at 31°C (89% positive) and 39°C (84% positive). (*C*) COS cells infected with VSV G33 for 5 hr at 31°C (77% positive) and 39°C (69% positive). (*D*) Analysis of COS cells stained for M with anti-VSV M protein antiserum (17% of the COS control cells are positive). COS cells were infected for 5 hr with VSV G31 at 31°C (15% positive) and 39°C (16% positive). A FACS II (Becton Dickinson) was used to quantitate the proportion of cells stained with monoclonal anti-G ascites (1:15 dilution) or anti-VSV M protein antisera (1:10 dilution). The stained cells were exposed to a laser light of 488 nm at an intensity of 200 MW. The fluorescence signal was determined with a photomultiplier potential of 650 V.

33°C and 39°C. Yet, as shown in Table 1, COS cells infected at 33°C were killed, while those at 39°C, expressing G but not M protein, were not killed. These experiments clearly indicate that expression of surface G protein is not sufficient for recognition and killing by NK cells and that the M protein, which is not expressed on the cell surface (Fig. 1*D*), has a significant influence.

DISCUSSION

Well-characterized ts mutants of VSV have proven useful in clarifying the viral proteins required for recognition by CTL. Zinkernagel *et al.* (9) reported that cells infected by the VSV G protein mutant (ts 045) were not killed by CTL at 39°C, although targets infected by the M protein mutant (G31) were. These results were confirmed by Hale *et al.* (19) with ts mutants. These and other studies established that the VSV G protein was the sole viral-encoded determinant required for recognition by CTL.

In attempting to bring the viral model to bear on the recognition of target structures by NK cells, several technical dif-

Table 2. Effect of anti-VSV or anti-IFN antisera on lysis of VSV-infected target cells

Antiserum	% specific lysis
None	23.9 ± 1.4
Anti-VSV	25.8 ± 1.1
Anti-VSV G	23.4 ± 1.6
Anti-VSV M	20.7 ± 0.9
Anti-IFN	26.5 ± 1.5

B16 melanoma cells were infected and incubated overnight at 31°C with VSV (wt) at a moi of 0.01; 50 μl of respective antibodies were added to target cells before the effectors. The ^{51}Cr-release assays were performed at 37°C at a lymphocyte-to-target ratio of 100:1, with an incubation period of 8–10 hr. Results are the average of triplicates in three independent experiments. Spontaneous release was 24%.

Table 3. NK binding of VSV-infected target cells

Target cells	Infection temperature, °C	% TBC
P815	31	4.3
	39	5.2
P815 + VSV (wt)	31	22.5
	39	22.5
P815 + ts G31	31	23.3
	39	2.9
P815 + ts 045	31	19.0
	39	3.7
P815 + VSV (wt)	31	21.7

The TBC assay was performed as described by Grimm and Bonavida (17) with minor modifications. The assay for cytotoxicity was performed as described (14). Nylon-wool nonadherent effector mouse spleen cells, obtained from mice treated with 100 μg of poly-rI:rC 24 hr previously were added to 4×10^5 target cells in 200 μl infected with virus at a moi of 3 and incubated for 3 hr (lymphocyte-to-target ratio of 1:2). After 10 min at 31°C, the cells were centrifuged at 200 × *g* for 5 min and resuspended in 60 μl for determination of the percentage of bound lymphocytes. The percentage of conjugating lymphocytes was determined by counting the number of lymphocytes bound to viable single-target cells (3–800 lymphocytes were counted).

Table 4. NK cell killing of COS cells transfected with pSVGL coding for the VSV G protein

COS cells	Cell sorted	G protein positive, %	Specific cytotoxicity, %
Uninfected	No	10	0
Infected with VSV ts G31	No	34	23
	Yes	85	25
Transfected with pSVGL	No	30	0
	Yes	77	0

COS cells were infected (31°C) and incubated overnight with the ts mutant G31 at a moi of 0.01. COS cells were transfected with pSVGL DNA (30 μg) in the presence of DEAE-dextran (500 μg/ml) in serum-free medium. After 3 hr, medium was replaced, and cells were incubated a further 40–45 hr. The cells were stained with monoclonal antibody to VSV G protein. Approximately 20–30% of the cells expressed surface G protein. To enrich for antigen-positive cells, the transfected cells were sorted in the FACS, and the percentage of G antigen-expressed cells used in the cytotoxicity assays was 80%.

ficulties intrinsic to the NK-cell system arose that were less problematic in the studies on CTL. While virus-infected targets are lysed within 4 hr by CTL, 8 hr are generally required for NK-cell killing of virus-infected targets. But since VSV is a lytic virus and rapidly inhibits host-cell synthesis, extended incubation times lead to unacceptably high spontaneous ^{51}Cr release from virus-infected cells. Conditions were chosen to obtain statistically significant killing without unacceptably high backgrounds (<30%).

As in the case of CTL, NK cells failed to bind or kill G-protein mutant (ts 045)-infected cells that failed to express the G protein on their cell surface at nonpermissive temperature. In contrast to CTL (9, 19) NK-cell killing could not be inhibited by antibodies to VSV. Rather unexpectedly, NK cells failed to kill two M-protein mutants at nonpermissive temperature, even though surface G protein could be detected by immunofluorescent staining and FACS analysis. The possibility that significant M protein is expressed on the outer surface of the plasma membrane and is recognized by NK cells is rendered unlikely by three observations: the G protein mutant at the nonpermissive temperature and tunicamycin-treated cells infected at permissive temperature (data not shown), both of which produce M protein, were not killed; and polyclonal anti-VSV antibodies highly reactive with M protein failed to block killing. These experiments formally indicate that, in contrast to CTL, expression of M and G proteins is required for lysis by NK cells. A critical test of this conclusion was made by transfecting the gene for the VSV G protein and expressing it in COS cells. After cell sorting, 80% of the cells expressed surface G protein but were totally refractory to lysis by NK cells.

These data lend themselves to two interpretations: (*i*) the surface G protein is the true target structure recognized by NK cells, but it must assume an appropriate conformation, which is imparted by its interaction with the M protein; and (*ii*) the insertion of the viral M and G proteins causes an alteration in the normal membrane structure, and it is this perturbed configuration of host constituents which is recognized by NK cells.

While it is not possible at present to discriminate between these two possibilities, both have considerable implications for understanding phenomena involving NK cells. If NK cells recognize conformational detemrinants, their mode of recognition would be different than that of T_H cells, which generally interact with linear or sequential determinants of antigens. If the second possibility obtains, it would be an interesting and important model of recognition of "altered self" rather than "self + X," and would endow NK cells with an unusual mode of recognition in the immune system. The second model would best explain the wide cross-reactivity of NK cells for a variety of targets—e.g., virus-infected, tumor, and embryonal cells and possibly antigen-presenting cells. We would speculate that recognition by NK cells may be mediated through lectin-like receptors that are nonclonally distributed but capable of initiating expansion of NK cells.

We express our appreciation to Drs. P. I. Marcus and H. Lodish for generously providing the VSV mutants used in these studies. We are also most grateful to Dr. B. Bonavida for his helpful advice on the target binding assay and providing us with pre-publication information. We are indebted to Drs. J. Holland, R. Weiss, H. Koprowski, and M. Pasternak for their kindness in providing antisera for these studies. We appreciate the assistance of Mrs. O. Harrish in performing the immunofluorescent staining and Mr. John Egan for technical assistance. The helpful support and discussion by Dr. L. Reid are gratefully acknowledged. This work was supported by Public Health Service Grants AI09807 and AI10702, Grant 1006 from the National Multiple Sclerosis Society, Irvington House Institute for Medical Research, and Grant BC301 from the American Cancer Society.

1. Herberman, R. B. & Ortaldo, J. R. (1981) *Science* **214**, 24–30.
2. Rager-Zisman, B. & Bloom, B. R. (1982) *Sem. Immunopathol.* **4**, 397–414.
3. Welsh, R. M., Jr. (1978) *J. Immunol.* **121**, 1631–1635.
4. Kiessling, R., Klein, E. & Wigzell, H. (1975) *Eur. J. Immunol.* **5**, 112–117.
5. Becker, S., Fenyo, E. M. & Klein, E. (1976) *Eur. J. Immunol.* **6**, 882–885.
6. Roder, J. C., Rosen, A., Fenyo, E. M. & Troy, F. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1405–1409.
7. Young, W. W., Durdik, E. M., Urdal, D., Hakamori, S. I. & Henney, C. S. (1981) *J. Immunol.* **126**, 1–6.
8. Pringle, C. R. (1975) *Curr. Top. Microbiol. Immunol.* **69**, 85–116.
9. Zinkernagel, R. M., Althage, A. & Holland, J. (1978) *J. Immunol.* **121**, 744–748.
10. Lodish, F. H. & Weiss, R. A. (1979) *Virology* **30**, 177–189.
11. Mather, J. P. & Sato, G. H. (1979) *Exp. Cell Res.* **124**, 215–221.
12. Gresser, I., Tovey, M. G., Bandu, M. T., Maury, C. & Brouty-Boye, D. (1976) *J. Exp. Med.* **144**, 1305–1323.
13. Rose, J. K. & Bergmann, J. E. (1982) *Cell* **30**, 753–762.
14. Minato, N., Bloom, B. R., Jones, C., Holland, J. & Reid, L. M. (1979) *J. Exp. Med.* **149**, 1117–1133.
15. Minato, N., Reid, L. M. & Bloom, B. R. (1981) *J. Exp. Med.* **154**, 750–762.
16. Ault, K. A. & Weiner, H. L. (1979) *J. Immunol.* **122**, 2611–2616.
17. Grimm, E. & Bonavida, B. (1979) *J. Immunol.* **123**, 2861–2869.
18. Rose, J. K. & Bergmann, J. E. (1983) *Cell* **34**, 513–524.
19. Hale, A. H., Witte, O. N., Baltimore, D. & Eisen, H. N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 970–974.