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Enhancement of Antibody-Dependent Cell-Mediated Cytotoxicity of Herpesvirus-Infected Cells by Complement

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An investigation was made of the effects of complement on the levels of antibody-dependent cytotoxicity (ADCC) mediated by bovine leukocytes against herpesvirus-infected target cells. Neutrophil-mediated ADCC was considerably enhanced upon the addition of low levels of complement that alone failed to induce lysis of antibody-sensitized target cells. This enhancement was most apparent under suboptimum conditions such as at low effector-to-target cell ratios, low levels of sensitizing antiserum, and short-duration assays. Furthermore, cells and classes of immunoglobulin unable to induce ADCC could do so in the presence of complement. The action of complement is considered in terms of a more tenacious bond formed between effector and target cells. The implications of the results are discussed in terms of the part that complement might play in enhancing antiviral recovery processes.

Antibody-dependent cytotoxicity (ADCC) is an in vitro phenomenon that can be demonstrated with various target cell systems and can be mediated by several types of effector cells (2, 3, 8, 11, 14, 17, 20, 23). Many suspect that ADCC forms a model of an in vivo mechanism of immunity (12, 13, 15, 19, 27), but this remains to be proven. All cells that mediate ADCC must bear a surface receptor for Fc (8), but, although the presence of such a receptor is necessary, it is not sufficient since cells bearing such receptors may fail to mediate ADCC against a particular target (4, 14, 17, 25). Similarly, union of effectors with targets by means of a different receptor, such as the complement receptor, may not result in cytotoxicity (10, 20, 21). However, the complement receptor may play a supportive role in ADCC (7, 18); this suggestion is further substantiated below. Why various cell types vary in their ADCC-mediating activity has not been adequately explained. It could depend upon the number, type, or affinity of Fc receptors, which in turn affects the tenacity of the bond to target cells. Alternatively, it may be the properties of the target cells themselves that largely determine whether or not ADCC occurs. We have adopted the hypothesis that target cell cytotoxicity only occurs if a sufficient bond is made between the effector and target cells. Such a union could be generated by bonds between the target cells and two types of surface receptors on the effectors; the Fc receptor and the complement receptor could act as candidates since both may be present on cells that mediate ADCC (A. S. Grewal, B. T. Rouse, and L. A. Babiuk, manuscript in preparation; 9, 10, 18, 20, 21). Using herpesvirus-infected target cells, we provide support for this hypothesis and show that the addition of complement below levels required for antibody-complement lysis considerably enhances ADCC and, furthermore, permits cells and classes of immunoglobulin (Ig) to mediate ADCC which in the absence of complement cannot do so.

(Some of the data presented in this article were published in preliminary form in a letter to Nature [London].)

MATERIALS AND METHODS

Cells used. Two populations of bovine cells were employed—polymorphonuclear neutrophils (PMN) collected, as described elsewhere, from mammary glands (22); and peripheral blood lymphocytes (PBL). The PMN were enriched by Ficoll-Hypaque flotation, consisted of 98 to 99% PMN, and were >99% viable as judged by trypan blue exclusion. The PBL were obtained by Ficoll-Hypaque flotation and were passed over glass wool columns equilibrated with RPMI 1640 containing 10% heat-inactivated autologous plasma. This procedure resulted in the removal of contaminating macrophages and neutrophils.

ADCC assay. Details of the ADCC assays, which were carried out in microtiter trays (no. 3040; Falcon Plastics, Oxnard, Calif.), were described previously (14). Assays were usually carried out for 6 h at 37°C in a humidified atmosphere of 5% CO_2. The culture medium used was RPMI 1640 containing 10% heat-inactivated fetal calf serum and buffered with 25 mM

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N-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES). At the assay termination, 50% of the supernatant fluids were collected for radioactivity measurements (counts per minute), and the percent specific ^{51}Cr release was computed by the formula:

$$\text{Specific release} = \frac{\text{CPM supernatant from test} - \text{CPM supernatant from control}}{\text{Total releasable CPM} - \text{CPM control}} \times 100$$

The total releasable counts per minute (CPM) was taken as that released from target cells in the presence of 3% Triton X-100. The controls contained either antibody-sensitized target cells in RPMI-HEPES, nonsensitized target cells in the presence of PMN or PMN plus complement, or nonsensitized target cells in the presence of RPMI-HEPES. These controls all released, nonspecifically, approximately the same quantity (±5%) of radioactivity. The target cells used were either normal Georgia bovine kidney cells or those infected 16 h before assay initiation with one plaque-forming unit per cell of infectious bovine rhinotracheitis (IBR) virus. The target cells were labeled at the time of infection with $Na_2{}^{51}CrO_4$ as described before (14). The target cells were removed from monolayers by brief trypsinization, washed three times, and added to wells that contained different concentrations of anti-IBR antibody. Dilutions of complement were added next, followed by different concentrations of effector cells. All tests were carried out in quadruplicate or sextuplicate, and all assays were performed at least three times. In the kinetics experiments, the reactants were subjected to brief centrifugation at 200 × *g* before incubation.

The anti-IBR serum used was from a bovine animal hyperimmunized against IBR virus. The preparation of IgM anti-IBR reagent was described elsewhere (16) and was kindly donated by T. K. S. Mukkur. Briefly, the reagent was obtained by sulfate precipitation followed by gel filtration (Bio-Gel P-300) and ion-exchange chromatography on triethylaminoethyl-cellulose. The preparation had a neutralization antibody titer against IBR virus of 1:20. The IgM fraction was free from IgG as judged by gel diffusion analysis and immunoelectrophoresis.

The complement used was rabbit complement obtained from Difco Laboratories, Detroit, Mich. The reagent was reconstituted from lyophilized material just before use and was diluted as required in RPMI-HEPES. Some reconstituted material was inactivated by heating at 56°C for 30 min.

Detection of Fc receptor-bearing cells. The technique for detecting F receptor-bearing cells was described previously (4). Briefly, effector cell populations were reacted with sheep erythrocytes coated with subagglutinating doses of rabbit anti-sheep erythrocyte serum for 30 min at 37°C. The number of rosette-forming cells was enumerated after the pellet was gently resuspended.

Detection of complement receptor-bearing cells. Complement receptors on PBL were detected as described by Arnaiz-Vellena et al. (1), and those on PMN were detected by the method of Huber and Wigzell (5). Sheep erythrocytes coated with subhemagglutinating doses of 19S rabbit anti-sheep erythrocyte serum (Cordis, Miami, Fla.) at 37°C for 30 min were reacted with fresh mouse serum (complement source) at a 1/20 final dilution for 30 min at 37°C. After three washes with RPMI 1640, cells were resuspended to 1% and mixed with an equal volume of purified PBL (4×10^6/ml). The mixture was spun for 5 min at 120 × *g*, after which the pellet was incubated at room temperature for 10 min and subsequently gently resuspended. Rosetting cells were enumerated.

For complement receptors on PMN, the method of Huber and Wigzell (5) was used. Briefly, zymosan (lot 24C-09419; Sigma Chemical Co., St. Louis, Mo.) was swollen by boiling in saline for 30 min; it was then washed twice in Hanks balanced salt solution and diluted to an estimated 1-mg/ml concentration in Hanks balanced salt solution. The beads were incubated with fresh mouse serum (1 mg of zymosan per ml for 0.1 ml of serum) for 30 min at 37°C, washed three times in Hanks balanced salt solution, and resuspended in 0.5 mg/ml. Rosettes were formed by reacting equal volumes of PMN at 4×10^6/ml with the zymosan-complement suspension for 1 min at 22°C. After centrifugation at 200 × *g* and additional incubation for 5 min at 22°C, the mixtures were gently agitated and rosettes were enumerated. Any cell with three or more beads adhering to the surface was recorded as a rosetting cell.

RESULTS

Antibody complement lysis of target cells. In the presence of a sufficient concentration of rabbit complement, the antibody-sensitized, IBR virus-infected target cells lysed (Fig. 1). As was to be expected, cytotoxicity was greatest with high concentrations of both complement and sensitizing antiserum. At a final complement concentration of 1/70 and an anti-IBR serum concentration of 1/800, the cytotoxicity level was close to the background level (in many

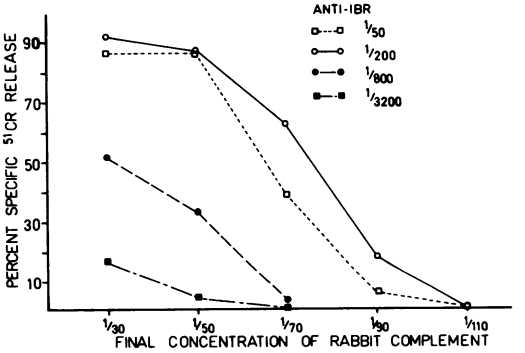

FIG. 1. *Lysis of anti-IBR-sensitized, IBR virus-infected Georgia bovine kidney cells at different rabbit complement concentrations. The antisera alone were not toxic to the target cells. Assays were performed for 6 h. Background release, 11.2%. Heat-inactivated complement failed to cause lysis of target cells at any antibody concentration.*

experiments it was at the background level). Since, as shown below, the anti-IBR concentration of 1/800 was more than sufficient to sensitize cells for PMN-mediated ADCC, these conditions were usually chosen to demonstrate complement-mediated enhancement of ADCC (ADCC-C).

Enhancement of PMN-mediated ADCC. We hypothesized that ADCC-C might best be demonstrated under conditions in which the extent of cytotoxicity is submaximal, i.e., at low effector-to-target cell ratios, low concentrations of sensitizing antiserum, and short-duration assays. The data in Fig. 2 to 4 support this assumption. The data in Fig. 2 compare the levels of cytotoxicity expressed by different numbers of PMN against antibody-sensitized target cells in the presence or absence of complement. At low ratios (6:1) of effectors to target cells, cytotoxicity could only be demonstrated when complement was present. However, as the effector-to-target ratio increased, the enhancement by complement was reduced. Thus, whereas the ratio of ADCC-C to ADCC at an effector-to-target ratio of 25:1 was 5:1, at 100:1 it was only 2.2:1. Not shown in Fig. 2 are controls demonstrating that lysis of virus-infected GBK cells by a 100:1 PMN-to-target cell ratio only occurred in the presence of anti-IBR serum and that uninfected GBK cells could not be lysed in the presence or absence of antiserum or of antiserum plus complement. Furthermore, the level of ^{51}Cr released from antibody-sensitized target cells in the presence of complement was not higher than the spontaneous release. Finally, heat-inactivated preparations of rabbit complement failed to enhance ADCC.

An example of the enhancement effect of complement at various concentrations of sensitizing anti-IBR serum is shown in Fig. 3. Whereas the degree of enhancement was marginal at high antibody concentrations, it increased as the antiserum concentration was reduced. In fact, although ADCC was not detectable at sensitizing-antibody dilutions greater than 1/1,600, ADCC-C was still demonstrable at a 1/12,800 concentration. At the highest antiserum concentration, some antibody-complement lysis of target cells was also detectable, but it was much lower than the cytotoxicity attributable to ADCC and ADCC-C.

Finally, the enhancement effect of complement was more apparent in short-duration assays (Fig. 4). Thus, only ADCC-C was detectable at h 1 of the assay and, as the assay time was increased to around 8 h, the differences between ADCC and ADCC-C cytotoxicity levels decreased. However, after 8 h the ratio of ADCC-C to ADCC remained approximately the same.

The experiments represented in Fig. 2 to 4 were repeated several times with essentially similar results. However, the actual ratio of ADCC to ADCC-C at a given antiserum concentration between different experiments was not constant.

Complement permits lymphocytes to express ADCC. In previous communications, we

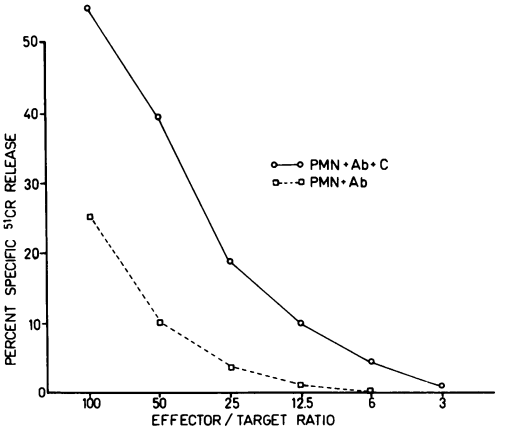

FIG. 2. *Effect of complement on PMN-mediated ADCC at different effector cell-to-target cell ratios. The anti-IBR concentration was 1/800 and the complement concentration was 1/70. In the absence of anti-IBR serum (but plus PMN), the level of release from infected target cells was the same as that from targets in the presence of medium alone or medium plus anti-IBR serum. The level of release from cultures with PMN, infected targets, and complement was at the same as the background. Assay time, 6 h. Background release, 12.8%. Heat-inactivated complement failed to enhance ADCC. PMN failed to mediate ADCC or ADCC-C against uninfected Georgia bovine kidney cells.*

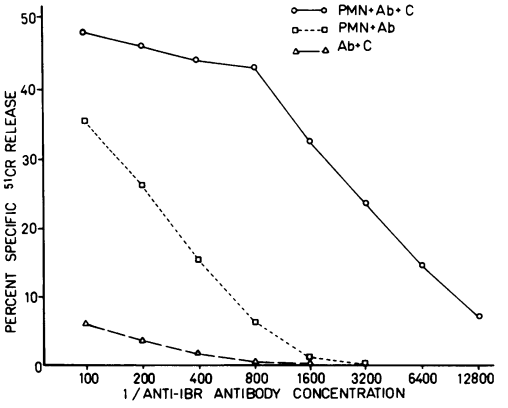

FIG. 3. *Effect of complement on PMN-mediated ADCC at different concentrations of anti-IBR-sensitizing antibody. Assay time, 6 h. Background release, 13.2%. PMN/target cell ratio, 100:1. Final concentration of complement, 1/70.*

have shown that, if PBL preparations are depleted of contaminating PMN and macrophages, they cannot mediate ADCC against antibody-sensitized, virus-infected target cells (4, 14). However, such lymphocyte preparations can express ADCC upon the addition of complement (Fig. 5). Approximately 25% of the purified PBL had the Fc receptor and 33% had the complement receptor.

Complement permits IgM antibody to mediate ADCC. As shown in other systems (5, 10, 21) and also expressed in Fig. 6, the IgM class of antibody is unable to mediate ADCC. Furthermore, the IgM anti-IBR preparation used failed to mediate significant levels of antibody complement lysis. This was true even at a 1/10 concentration of antiserum. However, upon the addition of complement to the ADCC reactants, a remarkable enhancement of cytotoxicity was apparent. Peak cytotoxicity was expressed at a 1/640 concentration of sensitizing IgM (same pattern in several separate experiments), and some cytotoxicity could still be demonstrated at a 1/40,960 dilution. A marked inhibition of cytotoxicity was routinely observed at high IgM concentrations. Whether the inhibition was attributable to anticomplementary effects of the IgM preparation or some steric hinderance effect was not investigated further.

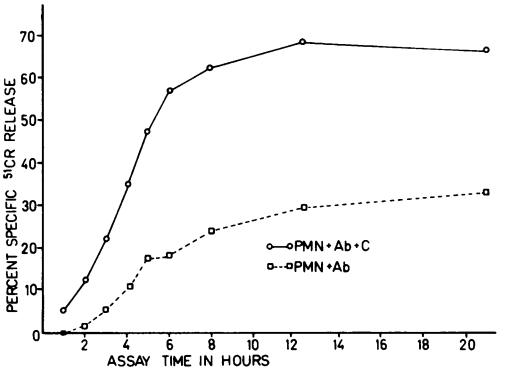

FIG. 4. *Effect of complement on PMN-mediated ADCC at different assay durations. Anti-IBR concentration, 1/800; complement concentration, 1/70. The background release from targets in the absence of complement was 6.7% at 1 h and 18.4% at 21 h. In the presence of added complement, the corresponding figures were 8.9 and 22%.*

DISCUSSION

ADCC can be demonstrated with a wide range of target cells and can be mediated by a variety of cell types, all of which must bear the surface receptor for Fc (2, 3, 8, 11, 14, 17, 20). Confusion has surrounded the identification of effector cells, but some of this confusion has occurred because effector cells are not equally adept at killing all target cells (4, 14, 17, 26). The reasons for the differences are unknown, but there are at least three possible explanations. (i) It could depend upon the extent of cytocidal effects generated by effector cells. (ii) It could be explained by the nature of the bond made between effectors and antibody-sensitized targets. (iii) It could be controlled solely by the target cell itself, with some cells perhaps undergoing more rapid repair than do others. In the present report, we have addressed ourselves to the second possibility and have shown that the type of bond between effectors and target cells can markedly influence

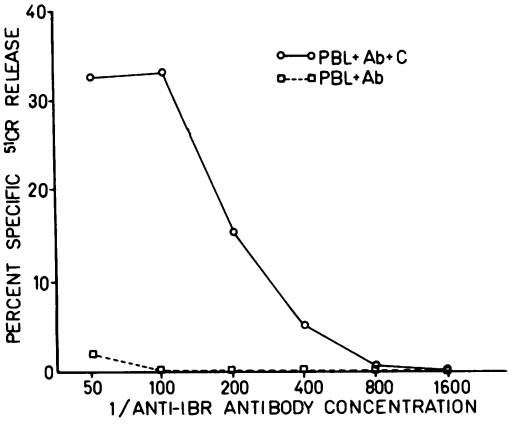

FIG. 5. *Effect of complement on PBL-mediated ADCC at different antibody concentrations. Assay time, 6 h. Background release, 11.9%. Complement concentration, 1/70. Similar results were observed at PBL-to-target cell ratios of 50:1 and 25:1, except that the cytotoxicity levels were lower.*

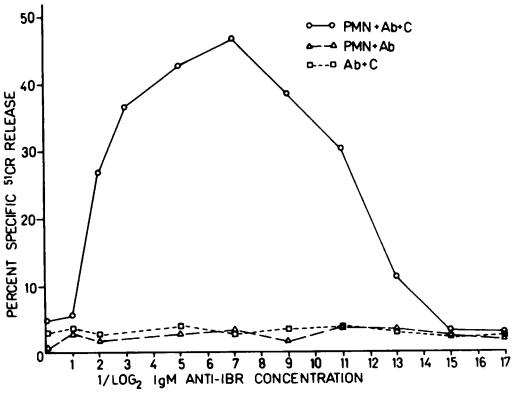

FIG. 6. *Effect of complement on IgM-mediated ADCC. Assay time, 6 h. Background release, 12.5%. Final complement concentration, 1/70. The IgM preparations were not toxic to target cells. Heat-inactivated complement failed to mediate cytotoxicity. The concentration at 1 $\log_2$ was 1/10.*

the degree of cytotoxicity. Thus, as shown by others (7) and confirmed here by using a virus-infected target cell system, the addition of a second bond between the effector cell and the target cell by means of complement results in enhancement of cytotoxicity over that mediated by the Fc receptor-Fc bond of classical ADCC. This effect was especially apparent under conditions of suboptimum cytotoxicity, such as at low effector-to-target cell ratios, low levels of sensitizing antiserum, and short-duration assays. In fact, in all three situations, conditions could be arranged whereby only complement-facilitated ADCC was demonstrable. It was of interest in this regard that cell types unable to mediate ADCC against viral targets could be made to do so by the addition of complement. Thus, lymphocytes that bear Fc receptors and can mediate ADCC against erythrocyte targets could not destroy herpesvirus-infected target cells (14). However, high levels of cytotoxicity resulted upon the addition of complement.

How complement facilitates ADCC is not known, but the formation of a more stable bond between effectors and targets or between sensitizing antibody and targets is a likely possibility. The idea of more tenacious bonds received support from the work of Scornik (18), who showed that, in the presence of complement, ADCC could be induced in the presence of inhibitory concentrations of IgG. His work did not, however, elucidate the actual mechanism whereby complement stabilizes the union between the ADCC components, nor did it show directly that effector cells were physically bound to target cells more tenaciously in the presence of complement than in its absence. It remains possible, in fact, that the additional complement bond signals the effector cell to generate a biochemical event that results in target cell destruction. This latter possibility is a need of investigation.

In contrast to the work of Lustig and Bianco (7), we demonstrated that complement also permitted a class of Ig otherwise unable to mediate ADCC to induce cytotoxicity. Thus, as shown in other systems (10, 21, 25), IgM antibodies could not mediate ADCC. However, upon complement addition, cytotoxicity was demonstrable even at high dilutions of sensitizing IgM. In fact, at high IgM concentrations, cytotoxicity was negligible but, with further dilution, levels of cytotoxicity rose to a peak (at a 1/640 dilution) and then declined. The inhibition at high IgM concentrations was perhaps attributable to steric hinderance, with the IgM blocking the binding of complement. The discordance between our results and those of others needs explanation, and the fact that we were dealing with a different target system, a different species, and a different class of effector cells makes the problem of explanation difficult. Furthermore, the Fc receptor known to be present on the herpesvirus-infected target cells themselves (6, 24; L. A. Babiuk and B. T. Rouse, unpublished data) might play some role in the system we have described. Clearly, additional experiments are required to elucidate why the IgM anti-IBR antibody can mediate ADCC in the presence of complement.

Although the present studies were performed entirely in vitro, it is interesting to speculate what possible relevance our findings have for understanding antiviral recovery mechanisms. It has been suggested that ADCC represents an in vitro model of an in vivo mechanism of antiviral defense (13, 15, 19). Since ADCC is enhanced by complement, this might be important for recovery, especially under marginal conditions such as at low levels of antibody and at times early in the virus-host cell interaction events when cells are expressing few viral antigens (15). It is tempting to speculate that perhaps, before episodes of herpesvirus recrudescent disease, complement levels are depressed, resulting in the function of a defense mechanism that restricts virus dissemination being impaired. These ideas can be evaluated experimentally.

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LITERATURE CITED

1. **Arnaiz-Villena, A., M. I. C. Gzongyossy, and J. H. L. Playfair.** 1974. Rosette-formation of mouse lymphocytes. II. T cells specification in a CRL subpopulation. Clin. Exp. Immunol. **18**:177–185.
2. **Gale, R. P., and J. Zigelboim.** 1975. Polymorphonuclear leukocytes in antibody dependent cellular cytotoxicity. J. Immunol. **114**:1047–1051.
3. **Greenberg, A. H., L. Shen, L. Walker, A. Arnaiz-Villena, and I. M. Roitt.** 1975. Characteristics of the effector cells mediating cytotoxicity against antibody coated targets. II. The mouse nonadherent K cell. Eur. J. Immunol. **5**:474–480.
4. **Grewal, A. S., B. T. Rouse, and L. A. Babiuk.** 1977. Mechanism of resistance to herpesviruses: comparison of the effectiveness of different cell types in mediating antibody-dependent cell-mediated cytotoxicity. Infect. Immun. **15**:698–703.
5. **Huber, C. H., and H. Wigzell.** 1975. A simple rosette assay for demonstration of complement receptor sites using complement coated zymosan beads. Eur. J. Immunol. **5**:432–435.
6. **Keller, R., P. Ricki, J. N. Goldman, and M. Goldman.** 1976. An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts. J. Immunol. **116**:772–775.
7. **Lustig, H. J., and C. Bianco.** 1976. Antibody-mediated cell cytotoxicity in a defined system: regulation by antigen, antibody and complement. J. Immunol. **116**: 253–260.
8. **MacLennan, I. C. M.** 1972. Antibody in the induction and inhibition of lymphocytotoxicity. Transplant. Rev.

13:67-90.
9. **Nussenzweig, V.** 1974. Receptors for immune complexes on lymphocytes. Adv. Immunol. **19**:217-258.
10. **Perlmann, P., H. Perlmann, and H. J. Muller-Eberhard.** 1975. Cytolytic lymphocytic cells with complement receptor in human blood: induction of cytolysis by IgG antibody but not by target cell bound C3. J. Exp. Med. **1**:287-296.
11. **Perlmann, P., H. Perlmann, and H. Wigzell.** 1972. Lymphocyte mediated cytotoxicity *in vitro*: induction and inhibition by humoral antibody and nature of effector cells. Transplant. Rev. **13**:91.
12. **Pollack, S., G. Heppner, R. J. Brawn, and K. Nelson.** 1972. Specific killing of tumor cells *in vitro* in the presence of normal lymphoid cells and sera from hosts immune to the tumor antigens. Int. J. Cancer. **9**:316-323.
13. **Rager-Zisman, B., and B. R. Bloom.** 1974. Immunological destruction of herpes simplex virus I infected cells. Nature (London) **251**:542-544.
14. **Rouse, B. T., R. C. Wardley, and L. A. Babiuk.** 1976. Antibody-dependent cell-mediated cytotoxicity in cows: comparison of effector cell activity against heterologous erythrocyte and herpesvirus-infected bovine target cells. Infect. Immun. **13**:1433-1441.
15. **Rouse, B. T., R. C. Wardley, and L. A. Babiuk.** 1976. The role of antibody dependent cytotoxicity in recovery from herpesvirus infections. Cell. Immunol. **22**:182-186.
16. **Rouse, B. T., R. C. Wardley, L. A. Babiuk, and T. K. S. Mukkur.** The role of neutrophils in antiviral defense—in vitro studies on the mechanism of antiviral inhibition. J. Immun. **118**:1957-1961.
17. **Sanderson, C. J., I. A. Clark, and G. A. Taylor.** 1975. Different effector cell types in antibody dependent cell-mediated cytotoxicity. Nature (London) **253**:376-378.
18. **Scornik, J. C.** 1976. Complement-dependent immunoglobulin G receptor function in lymphoid cells. Science **192**:563-565.
19. **Shore, S. L., A. J. Nahmias, S. E. Starr, P. A. Wood, and D. E. McFarlin.** 1974. Detection of cell dependent cytotoxic antibody to cells infected with Herpes simplex virus. Nature (London) **251**:350-352.
20. **Van Boxel, J. A., W. E. Paul, M. M. Frank, and I. Green.** 1973. Antibody-dependent lymphoid cell mediated cytotoxicity: role of lymphocytes bearing a receptor for complement. J. Immunol. **110**:1027-1036.
21. **Van Boxel, J. A., W. E. Paul, I. Green, and M. M. Frank.** 1974. Antibody dependent lymphoid cell mediated cytotoxicity—a role of complement. J. Immunol. **112**:398-403.
22. **Wardley, R. C., B. T. Rouse, and L. A. Babiuk.** 1976. The mammary gland of the ox—a convenient site for the collection of macrophage and polymorphs. J. Reticuloendothel. Soc. **19**:29-36.
23. **Wardley, R. C., B. T. Rouse, and L. A. Babiuk.** 1976. Antibody dependent cytotoxicity mediated by neutrophils: a possible mechanism of antiviral defense. J. Reticuloendothel. Soc. **19**:323-332.
24. **Westmoreland, D., and J. D. Watkins.** 1974. The IgG receptor induced by Herpes simplex virus: studies using radioiodinated IgG. J. Gen. Virol. **24**:167-170.
25. **Wisoloff, F., T. E. Michaelsen, and S. S. Froland.** 1974. Inhibition of antibody dependent human lymphocyte mediated cytotoxicity by immunoglobulin classes, IgG subclasses and IgG fragments. Scand. J. Immunol. **3**:29-38.
26. **Wunderlich, J., E. Rosenberg, J. Connolly, and J. Parks.** 1975. Characteristics of a cytotoxic human lymphocyte-dependent antibody. J. Natl. Cancer. Inst. **54**:537-542.
27. **Zisman, B., and A. L. Allison.** 1973. The role of antibody and host cells in the resistance of mice against coxsackie B-3 virus. J. Gen. Virol. **19**:329-335.