Species Source of Complement in Viral-Immune and Other Cytolytic Reactions[1] (34529)

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In a recent report (1) it was shown that murine ascites lymphoma cells infected with Newcastle disease or Sendai virus, and incubated for an appropriate period to allow emergence of the maturing virus at the cell membrane, were killed in the presence of antiviral rabbit serum and guinea pig complement. This reaction, designated viral-immune cytolysis, has been observed with other viruses infecting normal or neoplastic cells (2–4) and indicates antigenic conversion by the appearance of virus-induced antigens at the cell surface. Evidence for a similar phenomenon occurring *in vivo* and contributing to viral oncolysis has also been presented (1, 5, 6).

To demonstrate conclusively the essential role of complement in the cytolytic reaction it was, of course, necessary to inactivate the rabbit serum at 56° but this resulted in greatly reduced cytolysis when guinea pig complement was used. We now report that the heat-labile factor in the antiviral rabbit serum is replaceable by fresh normal rabbit serum which gives in certain immune cytolytic reactions results superior to those with guinea pig complement. Also, as measurements of cell death in immune cytolysis, dye exclusion and cell respiration will be compared.

Materials and Methods. An ascites lymphoma induced by Gross virus in C_3H/Bi mice and Krebs 2 ascites tumor cells propagated in Swiss mice were used in experiments on viral-immune cytolysis. Most of the procedures have been described in earlier publications (1, 5, 7). Sendai virus was grown in the allantoic sac of 11-day chick embryos. Rabbits and C_3H/Bi mice were immunized by repeated intramuscular or intraperitoneal injections of active virus in allantoic fluid. The resulting antisera had hemagglutination inhibition titers of 640 to 1280. Anti-parainfluenza-1 horse serum (HI titer 320 vs Sendai) and rabbit antiserum against boiled sheep cells (Forssman) were obtained commercially. Antisera against transplantation antigens of the C_3H/Bi ascites lymphoma were obtained by repeated inoculation into C_3H/HeJ mice of normal C_3H/Bi spleen followed by 10% tumor cell suspensions. C_3H/HeJ mice differ from the C_3H/Bi strain by one or two antigens other than the H2K group (5).

Guinea pig (GPC) and rabbit complement (RaC) were fresh normal sera stored frozen at −20 or −60°. Also lyophilized guinea pig complement was obtained commercially. Since the antisera described above were also stored frozen, it is likely that they contained residual components of complement or other heat-labile factors until inactivated at 56° for 30 min.

Cells were infected by incubation for 1 hr with Sendai virus in allantoic fluid diluted 1:20, (about 100 HA units/ml) and the virus removed by washing. Ascites lymphoma cells were incubated for an additional 18 hr in modified Eagle's medium (1) and the Krebs 2 cells in Hanks' balanced salt solution with 0.1% each of glutamate, lactate, and bovine albumin. Hemagglutination titers of cell-associated virus were 64 to 128. After being concentrated 2-fold in BSS with glumate, lactate, and bovine albumin the infected cells were incubated with antiserum and complement for 2–5 hr in roller tubes or the Warburg apparatus.

Dye exclusion tests were done with 0.1% toluidine blue (TB) added to equal parts of

[1] Aided by Grant CA-04965 from the National Cancer Institute, U. S. Public Health Service.

TABLE I. Comparison of Cytolysis with GPC and RaC in the Presence of Various Antiviral Sera.

Anti-Sendai serum[a]	Cells	Percentage of cells surviving with complement: No comp.-I[b] TB[c]	No comp.-I[b] O_2[c]	GPC-I TB	GPC-I O_2	RaC-I TB	RaC-I O_2	RaC-U[b] TB	RaC-U[b] O_2
Rabbit 1:10 inact.	Krebs 2	94	100	79	95	*10*	*29*	75	97
Rabbit 1:10 inact.	Ascites lymphoma	*57*	100	*65*	100	*6*	*26*	65	95
Rabbit 1:10 not inact.	Ascites lymphoma	*58*	76	*18*	*23*	*5*	*50*	50	85
Horse 1:5 inact.	Ascites lymphoma	74	74	70	58	*19*	86	—	—
Mouse 1:5 inact.	Ascites lymphoma	89	100	85	100	73	89	—	—
None	Krebs 2	90	(100)	93	100	66	90	83	97
None	Ascites lymphoma	83	(100)	80	100	70	85	65	90

[a] Hemagglutination inhibition titers of antisera:rabbit 1280, horse 320, mouse 640.

[b] I = cells infected, U = cells uninfected, complement dilution 1:20.

[c] TB = percentage of cells excluding toluidine blue; O_2 = rate of O_2 uptake as percentage of controls during hour 5 in the Warburg apparatus.

2% suspension of cells on a microscope slide and a coverslip placed over the drop. The percentage of cells stained was determined under a phase contrast microscope by counting 100-200 cells. Lysis was not an important factor unless most of the cells had already become stainable by TB. The effect of antiserum and complement on oxygen uptake was measured as the ratio:rate in the experimental group divided by the rate in the controls expressed as per cent.

Results. A comparison of GPC with RaC for cytolytic activity in the presence of antiviral sera from three species, rabbit, horse, and mouse is presented in Table I. By dye exclusion (TB) cell survival in the controls was 80–95%, and results in this range comprised infected and uninfected cells in the presence of guinea pig complement alone at 1:20. Rabbit complement was slightly cytotoxic at 1:20 and definitely so at 1:10 as measured by TB. Inactivated normal rabbit serum had no effect.

In the Warburg measurements, oxygen consumption of the controls declined slowly over a period of 5 hr as described previously (1). Cell survival as measured by O_2 uptake at the fifth hour was over 85% for uninfected cells incubated in the presence of inactivated antiserum and for infected and uninfected cells incubated with rabbit or guinea pig complement alone. In the absence of complement antiviral rabbit serum when not inactivated had a slight cytolytic effect against infected ascites lymphoma cells. With inactivated serum the addition of guinea pig complement did not significantly increase cytolysis, but rabbit complement caused a marked reduction in survival of Krebs 2 and ascites lymphoma cells both by TB and O_2. When the antiviral rabbit serum had not been heated to 56° both guinea pig complement, as previously reported (1), and rabbit complement augmented the cytolytic effect. Dye exclusion with uninfected cells, rabbit complement, and antiviral sera (last two columns of Table I) indicated a slight killing effect, but this was not evident in O_2 uptake. With antiparainfluenza-I horse serum, some cytolysis, most evident by TB, was observed. The results with anti-Sendai mouse serum are preliminary but indicate little cytolytic activity even though the number of HI units used in the test was comparable to the other sera.

A comparison of the results of dye exclusion and O_2 uptake as presented in Table I reveals several instances in which a portion of the cells apparently became permeable to toluidine blue but continued to respire. Dye exclusion was below the minimum control level of 80% but O_2 uptake as percentage of the controls was above 90%. With RaC alone or with uninfected cells, the results suggested a nonspecific effect of the heterologous serum on the cell membrane detected by TB but not by O_2 uptake.

TABLE II. Dye Exclusion with Guinea Pig Complement and Antiserum (Not Inactivated) of Krebs 2 Ascites Cells Infected with Sendai: Effect of Heated Normal Rabbit Serum.

Rabbit antiserum	Cells[a]	No compl.	GPC 1:20	GPC +RaCh 1:10[b]	GPC +RaCh 1:40
1:10	I	*5*	*<2*	*<2*	—
	U	78	<2	<2	—
1:20	I	*45*	*15*	*2*	*<2*
	U	72	81	66	72
1:40	I	82	*12*	*8*	*<2*
	U	85	81	71	90
1:100	I	80	90	*34*	*54*
	U	—	97	98	97
None	I	72	92	53	80
	U	—	97	92	95

[a] I = infected cells; U = uninfected cells.

[b] RaCh = Normal rabbit serum heated 56°, 30 min.

When not inactivated, anti-Sendai rabbit serum by itself at dilutions of 1:10 to 1:20 was cytolytic for Krebs 2 cells infected with Sendai virus but not for uninfected cells (Table II). However, when guinea pig complement was added, marked cytolysis was observed at 1:10 antiserum with normal as well as infected cells. Since this was not observed with inactivated serum (Table I), the presence of a labile nonspecific factor is indicated. Dilution of the antiserum eliminated this nonspecific effect and augmentation of cytolysis by GPC was demonstrable with noninactivated antiserum at dilutions of 1:20 and 1:40 but not 1:100.

Effect of heated normal rabbit serum. A recent report (8) indicated that inactivation of rabbit serum at 56° produces an inhibitor that blocks the hemolytic action of GPC on sensitized erythrocytes. To test for this inhibitor against GPC with Sendai-infected cells and antiserum, an excess of heated normal rabbit serum (Ra Ch) was added to tubes containing various dilutions of antiserum and GPC 1:20. The results are presented in the two right-hand columns of Table II. At antiserum dilutions of 1:20 and 1:40, where partial cytolysis occurred in the presence of GPC, addition of Ra Ch did not reduce cytolysis. In fact, Ra Ch appeared to augment slightly the action of GPC, and this effect was still evident with Ra Ch diluted 1:40.

Complement source in other cytolytic reactions. Sera from C_3H/HeJ mice that had received repeated doses of 10% ascites lymphoma cells originating in the genetically closely related C_3H/Bi strain were tested for cytolysis with the lymphoma cells. The results presented in Table III demonstrate strong cytolysis in the presence of RaC but no effect of GPC. Absorption with ascites lymphoma cells removed the antibodies. At lower serum dilutions agglutination of the lymphoma cells was observed both in the presence and absence of complement. These results suggest that cytolytic reactions using RaC may be developed as a sensitive method for studying histocompatibility and possibly tumor antigens.

Recent reports of changes in Forssman antigen reactively resulting from virus infection (9) led to an examination of infected and uninfected tumor cells using the cytolytic reaction with anti-F rabbit serum. No significant change of Forssman reactivity due to infection with Sendai virus was observed, but the results were of interest because neither GPC nor RaC increased this moderate cytolytic reaction. Absorption of the antiserum with ascites lymphoma cells removed its reactivity. Again less effect on O_2 uptake than on dye exclusion was observed (Table III) suggesting that primary damage is on the cell membrane. Krebs 2 ascites cells seemed

TABLE III. GPC and RaC in Cytolytic Reactions of Histocompatibility and Forssman Antigens in Ascites Lymphoma Cells.

Antiserum[a]	Dilution	Test	Percentage of cells surviving with complement: No comp.	GPC 1:20	RaC 1:20
C_3H/HeJ mouse	1:10	TB	80	85	2
Anti-C_3H/Bi	1:40	TB	—	—	2
	1:160	TB	—	—	40
No serum	—	TB	—	90	67
Rabbit					
Anti-Forssman	1:20	TB	20	12	19
Absorbed[b]	1:20	TB	90	88	—
No serum	—	TB	92	70	80
Anti-F	1:20	O_2	68	79	56
No serum	—	O_2	—	98	85

[a] All sera inactivated at 56°.

[b] Serum absorbed with ascites lymphoma cells.

somewhat less sensitive than ascites lymphoma to cytolysis by the anti-F serum.

Titrations of hemolysis of sensitized sheep cells with GPC and RaC gave an endpoint of 160 for GPC as compared with 20 for RaC against the same sensitized cells. The low activity of RaC in this hemolytic system is in accord with observations of others (8).

Discussion. The superiority of RaC over GPC has been demonstrated in other lytic reactions. Valentine *et al.* (10) found increased liberation of histamine from mast cells by rabbit antiserum in the presence of RaC as compared with GPC. Rat complement was about equal to RaC and human complement intermediate in efficiency. With the immune globulin fraction of the rabbit antiserum, GPC had the same effect as RaC. In our experiments GPC influenced viral immune cytolysis only when the antiserum had not been heated but no inhibitor of GPC, such as that found in lysis of sensitized erythrocytes (8), could be demonstrated in heated normal rabbit serum. The alternative interpretation that a heat-labile factor in the antiserum (stored at —60°) supplements the action of GPC (1) is therefore still open. Fractionation of the antiserum, which remains to be done, would, no doubt, help to answer this question.

Since antiviral mouse serum failed to produce significant cell destruction even with RaC, another mechanism for the enhanced *in vivo* oncolysis of Sendai-infected ascites lymphoma cells in virus-immune mice (1) must be considered. This might involve the action of virus-immune lymphocytes instead of antibody and complement. However, mouse serum can be cytolytically reactive with RaC as demonstrated in the experiments with histocompatibility antigens. The failure of either RaC or GPC to augment the killing effect of Forssman antiserum on ascites cells suggests that the reactions of the F antigen at the cell membrane may be of a different nature from those of viral or H antigens.

A comparison of dye exclusion and respiration as measures of the killing effect of antibody and complement indicates that under some conditions cells may become permeable to toluidine blue before impairment of respiration. This would be consistent with a primary effect on the cell membrane and temporary maintenance of O_2 uptake by cytoplasmic components. This sequence of events is the opposite of that previously reported (7) for nutritional depletion, chemical poisons, and viral infection in Ehrlich ascites cells where decline in respiratory activity preceded the appearance of permeability to eosin. In these earlier studies only *Cl. welchii* toxin

(lecithinase) showed an effect resembling that of antibody and complement on dye exclusion and respiration.

Summary. Fresh normal rabbit serum was more effective than guinea pig complement in immune cytolytic reactions with inactivated antiviral serum and ascites tumor cells infected with Sendai virus, and also in cytolysis with a mouse histocompatibility antigen and homologous antibody. When the antiviral rabbit serum had not been heated to 56° both guinea pig complement and rabbit complement augmented viral-immune cytolysis. A primary effect of immune cytolysis on the cell membrane was indicated by the appearance in some cells of permeability to dye before respiration was impaired.

1. Eaton, M. D. and Scala, A. R., Proc. Soc. Exp. Biol. Med. **132**, 20 (1969).

2. Stuck, B., Old, L. J., and Boyse, E. A., Nature **202**, 1016 (1964).

3. Wiktor, T. J., Kuwert, E., and Koprowski, H. J., Immunol. **101**, 1272 (1968).

4. Holmes, K. V., Klenk, H. D., and Choppin, P. W., Proc. Soc. Exp. Biol. Med. **131**, 651 (1969).

5. Eaton, M. D., Levinthal, J. D., and Scala, A. R., J. Nat. Cancer Inst. **39**, 1089 (1967).

6. Hamburg, V. P. and Svet-Moldavsky, G. J., Nature **203**, 772 (1964).

7. Eaton, M. D., Scala, A. R., and Jewell, M., Cancer Res. **19**, 945 (1959).

8. Kempf, R. A., Gigli, I., and Austen, K. F., J. Immunol. **102**, 796 (1969).

9. O'Neill, C. H., J. Cell Sci. **3**, 405 (1968).

10. Valentine, M. D., Bloch, K. J., and Austen, K. F., J. Immunol. **99**, 98 (1967).

Received Oct. 17, 1969. P.S.E.B.M., 1970, Vol. 133.