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Human antibody-dependent cell-mediated cytotoxicity against target cells infected with respiratory syncytial virus

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SUMMARY

A chromium release assay was established to study human antibody-dependent cell-mediated cytotoxicity (ADCC) of HEp 2 cells infected with respiratory syncytial (RS) virus. Human peripheral blood lymphocytes in the presence of specific antibody to RS virus caused *in vitro* lysis of RS virus infected target cells. ADCC was detected in sera of infants with RS virus infections and in specimens of colostrum. The ability of serum or colostrum to mediate the cytotoxic reaction appeared to be related to the level of specific IgG, or IgA antibody to RS virus, as detected by membrane fluorescence. Separation of effector cells by their glass adherence properties showed that the ability to produce cytotoxicity resided in non-adherent effector cells.

INTRODUCTION

Lysis of virus-infected cells by immune reactions may play a significant role in both host defence and in the production of pathological lesions during viral infection (Allison, 1972). Mechanisms which may be responsible for lysis of virus-infected cells include the cytolytic action of antibody and complement (Smith *et al.*, 1972), the interaction of antibody, complement and leucocytes (Lodmell *et al.*, 1973) and lysis by immune cytotoxic lymphocytes (Steele *et al.*, 1973). A further mechanism, which until recently was only applied to non viral systems, is antibody-dependent cell-mediated cytotoxicity (Perlmann & Holm, 1969; MacLennan, Loewi & Howard, 1969).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an immune process in which specific antibody acts synergistically with non immune lymphoid cells to produce target cell lysis. Recently, *in vitro* assays for ADCC have been described for a number of virus-cell systems. Herpes virus has been studied in human (Shore *et al.*, 1974; Russell *et al.*, 1975), murine (Rager-Zisman & Bloom, 1974; Ramshaw, 1975) and bovine (Rouse *et al.*, 1976) cell systems. ADCC has also been described for mumps (Harfast *et al.*, 1975) and Epstein–Barr virus (Pearson & Orr, 1976; Jondal, 1976) infection in humans and Moloney sarcoma virus infection in mice (Lamon *et al.*, 1975).

The present investigation was concerned with establishing an *in vitro* ADCC assay for respiratory syncytial (RS) virus-infected HEp 2 cells, using human antibody and effector cells. This virus was chosen as it has been shown to be the chief viral pathogen in respiratory infections of infancy (Channock *et al.*, 1961; Gardner, 1968). Severe infection with RS virus occurs predominantly in infants between 2 and 6 months of age. At this time, moderate to high levels of maternally derived serum antibody are present in the infants' sera, but appear to provide little protection against severe lower respiratory tract infection with RS virus. We present evidence that antibodies in the sera of infants with RS virus infections were able to act synergistically with non immune lymphoid cells to mediate in vitro lysis of RS virus infected target cells, and discuss the possible *in vivo* significance of this immune reaction.

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MATERIALS AND METHODS

Virus. Long strain RS virus was inoculated onto HEp 2 cells and, after several passages, the resulting virus suspension was divided into 1-ml aliquots and stored at −70°C until required. The virus pool was titrated on HEp 2 cells and a titre of $TCD_{50}10^{-5.5}$ was recorded.

Sera and colostra. Specimens of sera were taken from patients with respiratory infections on admissions to hospital and one to two weeks later and stored at −20°C. The colostra specimens were taken from a series of women admitted to the Princess Mary Maternity Hospital in Newcastle-upon-Tyne (Downham *et al.*, 1976). Colostra were also stored at −20°C until required. Both sera and colostra were examined for specific IgA/IgM/IgG antibodies to RS virus by a membrane fluorescent antibody technique described elsewhere (Scott *et al.*, 1976). All sera and colostra were heat-inactivated at 56°C for 30 min before being used in the ADCC assay.

Effector cells. Peripheral venous blood was obtained from ten different donors who were laboratory personnel. Twenty millilitres of blood was defibrinated with glass beads and lymphocytes were separated using Ficol–sodium metrizoate solution (Lymphoprep Nyegaard). Yields ranged from $0{\cdot}5–1\times10^6$ mononuclear cells per 1 ml of defibrinated blood with a viability of not less than 90% and lymphocyte purity of 95%.

ADCC assay. Confluent monolayers of HEp 2 cells (approximately 5×10^6 cells) were grown in Falcon tissue culture bottles and were inoculated with 1·5 ml of a 1/5 dilution of the RS virus stock. Following 3 days incubation at 37°C the cells were washed twice with Earle's balanced salt solution and exposed to 200 μCi ^{51}Cr (Sodium chromate, Radiochemical Centre, Amersham) in a final volume of 3 ml RF 10 medium for a period of 60 min at 37°C. The labelled cell monolayer was washed eight times with 5 ml amounts of Earle's balanced salt solution. The cells were removed from the bottles by incubation for 2 min at 37°C in 3 ml of a solution of 0·25% trypsin diluted 1/3 in versene. The resulting cell suspension was washed in RF 10 medium and resuspended in the same medium at a concentration of 5×10^4 cells/ml. Uninfected HEp 2 cells were treated in exactly the same way.

The cells were dispensed into flat-bottomed wells of a Falcon Microtest II tissue culture plate at 100 μl per well. Fifty microlitres of the serum/colostrum dilution were added and the plates incubated at 30°C for 30 min. One hundred microlitres of the effector cell suspension (2×10^6 cells/ml for an effector-target cell ratio of 40:1) were added, the plates centrifuged at 500 r.p.m. for 5 min and subsequently incubated at 37°C in an atmosphere of 5% CO_2+air for 4 hr. At the end of the incubation period 100 μl of supernatant was withdrawn from each well and the radioactivity determined in an LKB 80000 gamma sample counter. Triplicate determinations were performed on all reported data.

The amount of ^{51}Cr taken up by the target cells was determined in a 100-ml sample directly and the result was multiplied by 1/2·5 to allow for the dilution of target cells with effectors and antibody in the wells of the plate. In order to assess the maximum available ^{51}Cr release 100 μl of targets were incubated with 150 μl 5% Decon in each experiment. The maximum percentage ^{51}Cr release available was then calculated from the radioactivity in a 100-μl sample of the Decon release divided by the amount of ^{51}Cr taken up by the target cells. This was usually 90% or more. Percentage ^{51}Cr release due to ADCC was calculated from the following equation:

$$\frac{\text{release from infected target cells+antibody+effectors}}{\text{Maximum available release with 5\% Decon.}}\times 100.$$

Percentage specific ^{51}Cr release due to ADCC was calculated from the following equation:

$$\frac{\text{release from infected target cells+antibody+effectors}-\text{spontaneous release from infected target cells alone}}{\text{Maximum available release with 5\% decon}-\text{spontaneous release from infected target cells alone}}\times 100.$$

Percentage ^{51}Cr release and percentage specific ^{51}Cr release for infected target cells and effectors without antibody were calculated for each experiment. Non-infected target cell controls were also included in each experiment.

Absorption of serum with RS virus infected cells. 0·5 ml of a 1/500 dilution of a serum, containing high levels of specific RS virus antibody, was mixed with 2×10^7 RS virus infected HEp 2 cells. The mixture was incubated at 4°C for 60 min and subsequently centrifuged to remove the cells. The same procedure was repeated using uninfected HEp 2 cells. The supernatants were then tested in the ADCC assay at an effector-target ratio of 40:1 and an incubation period of 4 hr.

Removal of adherent cells. Mononuclear effector cells, previously prepared by Ficol–sodium metrizoate separation, were further purified by removing adherent cells using the technique described by Alderson *et al.*, 1976. Adherent cells were recovered by mild enzymatic treatment of the glass surface with a solution of 0·25% trypsin diluted 1/3 in versene and the two cell populations were washed in RF 10 medium and resuspended at a concentration of 2×10^6 cells/ml.

RESULTS

Spontaneous release of ^{51}Cr and expression of RS virus antigen

Preliminary experiments were performed to determine the temporal relationship between the appearance of RS virus antigen on the surface of HEp 2 cells and the capacity of the cells to retain ^{51}Cr. The presence of RS virus surface antigen at 1–5 days after infection was detected by the membrane fluorescent

TABLE 1. Comparison of percentage spontaneous ^{51}Cr release from and presence of RS virus membrane antigen in HEp 2 cells

Days after RS virus infection	Percentage spontaneous ^{51}Cr release after 4 hr incubation	Percentage cells exhibiting RS virus membrane fluorescence
0	21±0·4*	0
1	23±0·6	15
2	20±0·3	30
3	21±0·7	75
4	25±0·5	95
5	45±0·7	95

* ± s.e.

antibody technique (Scott *et al.*, 1976). In parallel experiments, RS virus-infected HEp 2 cells were labelled with ^{51}Cr and the amount of isotope release was determined after an incubation period of four hours. The results are shown in Table 1, from which it can be seen that optimal conditions of virus expression and ^{51}Cr retention were obtained 3–4 days after infection. Three days RS virus-infected cells were used, therefore, throughout the period of investigation.

A comparison was also made of the spontaneous isotope release from RS virus infected cells and non-infected HEp 2 cells in twenty-one independent experiments, performed throughout the period of investigation. Percentage spontaneous ^{51}Cr release after 4 hr incubation was found to be 19 (±2)% and 20 (±3)% respectively.

Optimum conditions for ADCC

A series of experiments were undertaken to study the kinetics of the cytotoxic reactions involved in the ADCC assay for RS virus. Fig. 1 shows the relationship of percentage ^{51}Cr release to incubation time when effector cells were reacted with RS virus infected HEp 2 cells, labelled with ^{51}Cr, in the presence or absence of specific antibody to RS virus. The effector-target cell ratio was 40:1.

Specific antibody-mediated ^{51}Cr release was first observed after 2 hr incubation and became maximal between 4 and 7 hr incubation. Further incubation led to an increase in spontaneous ^{51}Cr release in the absence of antibody.

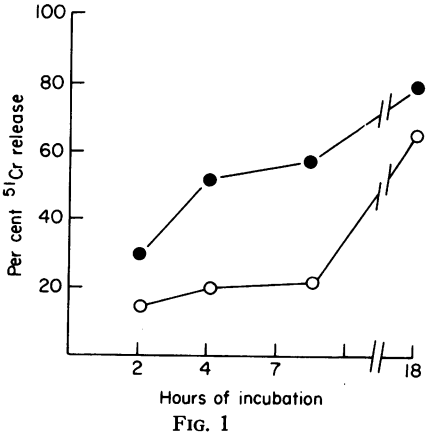

FIG. 1

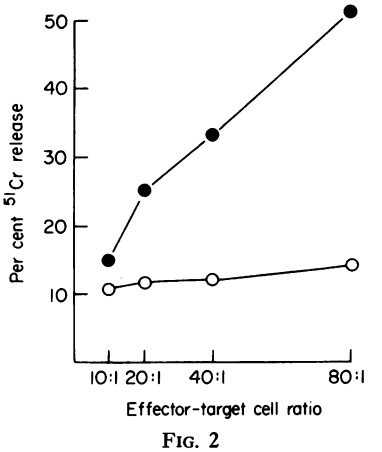

FIG. 2

FIG. 1. Kinetics of the ADCC assay. Effector cells were incubated with RS virus-infected target cells at a ratio of 40:1 in the presence (●) and absence (○) of specific antibody to RS virus.

FIG. 2. Effect of effector-target cell ratio on the ADCC assay. A constant number (5×10^3) of RS virus-infected target cells were incubated with increasing numbers of effector cells in the presence (●) and absence (○) of specific antibody to RS virus.

TABLE 2. Comparison of percent specific ^{51}Cr release and presence of IgA, IgG and IgM antibody to RS virus by membrane fluorescence in sera

Serum no.	* Titre of specific RS virus antibody by membrane fluorescence			Percent specific ^{51}Cr release in ADCC assay		Difference
	IgA	IgG	IgM	Serum present (A)	Serum absent (B)	A–B
0837	1/100	1/6000	1/10	47·0	17·2	+29·8
7159	1/40	1/1600	1/20	34·7	−2·8	+37·5
7070	1/40	1/100	< 1/10	29·8	5·7	+24·1
5113	†< 1/10	1/100	1/10	15·6	−2·8	+18·4
7071	< 1/10	< 1/10	< 1/10	2·3	−2·8	+ 5·1
9774	< 1/10	< 1/10	< 1/10	8·2	8·4	−0·2
5666	Neg	Neg	Neg	−1·4	16·9	−18·3
9886	Neg	1/10	Neg	12·3	16·9	−4·6

Neg = negative.

* Titre = highest dilution exhibiting + → ++ fluorescence.

† < 1/10 titre = + or trace fluorescence at a dilution of 1/10.

The effect of adding an increasing number of effector cells to a fixed number (5×10^3) of RS virus infected target cells is shown in Fig. 2. The effect was studied in the presence and absence of specific antibody to RS virus and the percentage ^{51}Cr release was determined after an incubation period of 4 hr. Specific antibody-mediated ^{51}Cr release was detected at an effector–target cell ratio of 10:1 and rapidly increased up to a ratio of 80:1.

Throughout the remainder of the study, the ADCC assay was performed with an effector-target cell ratio of 40:1 and an incubation period of 4 hr. An effector-target cell ratio of 80:1 was not used routinely as it proved uneconomical for the number of lymphocytes available.

Cytotoxicity mediated by RS virus antibody positive sera and colostra

A series of eight serum specimens from infants with respiratory infections were tested at an initial dilution of 1/10 in the ADCC assay (final dilution in plate 1/50). The sera were also examined for the presence of specific IgA, IgG and IgM antibody to RS virus by membrane fluorescence (Table 2). The

TABLE 3. Comparison of percent specific ^{51}Cr release and presence of IgA, IgG and antibody to RS virus by membrane fluorescence in colostra

Colostrum no.	Titre of specific RS virus antibody by membrane fluorescence			Percent specific ^{51}Cr release in ADCC assay		Difference A–B
	IgA	IgG	IgM	Colostra present (A)	Colostra absent (B)	
9747	1/20	1/80	Neg	33·1	16·1	+17·0
9290	1/60	1/40	Neg	29·0	16·1	+12·9
9411	1/200	1/20	Neg	25·2	16·1	+9·1
9402	1/80	1/20	Neg	19·1	16·1	+3·0
9287	1/10	1/10	Neg	13·3	16·1	−2·8
9504	1/20	< 1/10	Neg	20·7	16·1	+4·6

Neg = negative.

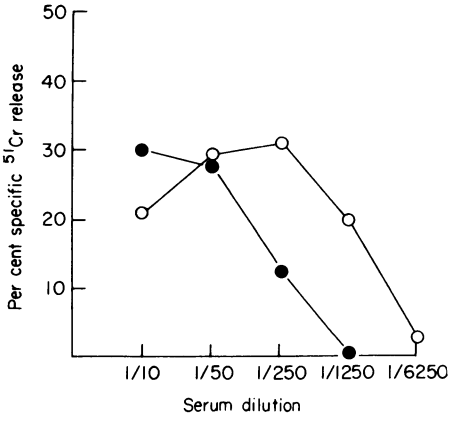

FIG. 3. Effect of serum dilution on the ADCC assay. RS virus-infected target cells were incubated for 4 hr at an effector–target cell ratio of 40:1 with increasing dilutions of serum 4760 (●) and serum 2299 (○).

variation in the percentage specific ^{51}Cr release in the absence of serum was a result of the specimens being assayed in different experiments. A comparison of the specific ^{51}Cr release in the presence and absence of serum, however, showed that a positive result was obtained for five of the eight sera examined (Table 2). Four of the five sera exhibiting positive cytotoxicity were found to possess detectable IgG antibody to RS virus at a titre of 1/10 or greater, as detected by membrane fluorescence. Three of the five sera also possessed detectable IgA or IgM at a titre of 1/10 or greater, the level of positive cytotoxicity (+5·1%) in specimen 7071, which contained IgA, IgG and IgM antibody to RS virus at a titre of <1/10, was considerably lower than the levels observed in the four other positive specimens. Furthermore, the level of cytotoxicity in the sera appeared to show a closer correlation to the level of IgG antibody than to IgM or IgA, as detected by membrane fluorescence.

A series of colostra specimens were also tested in the ADCC assay and for the presence of specific IgA, IgG and IgM antibody to RS virus by membrane fluorescence (Table 3). The percentage specific ^{51}Cr release in the absence of colostrum was 16·1 for each specimen, of colostrum, as they were all tested in the same experiment. Five of the six specimens gave a positive result on comparison of the specific ^{51}Cr release in the presence and absence of colostrum (Table 3). All six specimens of colostra, exhibiting positive cytotoxicity, possessed IgA antibody at a titre of 1/10 or greater as detected by membrane fluorescence. Five of the six colostra also contained IgG antibody at a titre of 1/10 or greater. Specific IgM antibody to RS virus was undetectable by membrane fluorescence in any of the six specimens of colostra examined.

Effector cells were obtained from ten different donors throughout the period of investigation. As can be seen from Table 2, there was a variation in the level of percentage specific ^{51}Cr release in the absence of serum. This variation in the level of cytotoxicity of RS virus infected targets with effector cells alone was found with different donors and at different times with the same donor. Furthermore, high levels of

TABLE 4. Effect of different effector cell populations in the ADCC assay

Effector–target cell ratio	Percent ^{51}Cr release with effector cells: FICOL–metrizoate only	Non-adherent cell population	Adherent cell population
40:1	30 (±0·7)*	40 (±0·6)	1·2 (±0·6)
20:1	12 (±0·6)	17 (±0·5)	0·6 (±0·3)
10:1	4 (±0·3)	8 (±0·3)	1·1 (±0·4)

* ±s.e.

cytotoxicity of RS virus-infected target cells with effector cells alone were usually accompanied by similar levels of cytotoxicity of uninfected target cells with effector cells. In each experiment, however, the level of cytotoxicity observed with uninfected target cells and effectors in the presence of serum/colostrum was the same as that with uninfected target cells and effectors in the absence of serum/colostrum.

The cytotoxicity resulting from adding increasing dilutions of sera, with high levels of RS virus antibody, was studied in the ADCC assay. Two patterns of response were recorded for sera, which contained specific IgG antibody to RS virus at a titre of 1/1000 or greater. An example of each type of response is shown in Fig. 3. The percentage specific ^{51}Cr release in the absence of serum for the two examples shown was 8·7 for 2299 and 5·9 for 4760. The prozone phenomenon observed with 2299 was recorded for 50% of the sera examined.

Absorption of serum with RS virus-infected cells

Support for the specificity of the ADCC assay was obtained by showing that absorption of a serum containing a high titre of RS virus antibody with RS virus-infected HEp 2 cells abrogated cytotoxicity to RS virus infected targets ($25 \pm 0{\cdot}8\%$ ^{51}Cr released using an unabsorbed serum and $1{\cdot}6 \pm 0{\cdot}5\%$ ^{51}Cr release using absorbed serum). Absorption using uninfected HEp 2 cells did not affect cytotoxicity ($27 \pm 0{\cdot}06\%$ ^{51}Cr release).

Removal of adherent cells

The cytotoxic potential of adherent and non-adherent populations of effector cells in the ADCC assay was investigated in a series of experiments. The cytotoxic activity of the original effector cell population and the adherent and non-adherent cells was compared at different effector-target cell ratios with a serum containing a high RS virus antibody titre (Table 4).

The ability to produce cytotoxicity of RS virus infected target cells was found to reside in the non-adherent effector cell population and the level of cytotoxicity produced was found to be higher than that observed with the original effector cell population. Cytotoxicity was not observed when the adherent effector cell population was tested in the ADCC assay.

DISCUSSION

The present investigation has shown that sera, containing specific antibody to RS virus, were able to mediate *in vitro* cytotoxicity of HEp 2 cells, exhibiting RS virus membrane antigen, in the presence of normal peripheral blood lymphocytes. This report extends to the RS virus system the phenomenon of ADCC, which has already been demonstrated in a number of other virus systems. ADCC assays, involving human antibody, effectors and target cells, have been established for type 1 and 2 herpes simplex virus (Shore *et al.*, 1976) and Epstein–Barr virus (Jondal, 1976).

The RS virus system shares some properties previously described for ADCC in other virus and tumour systems; no requirement for complement, prozone phenomenon exhibited by sera and effector cells which are nonadherent to glass surfaces (MacLennan, 1972; De Landazuri, Kedar & Fahey, 1974). The results from the present study indicate that the classical complement sequence is not involved in the ADCC reaction, as heat-inactivated serum effectively mediated cytotoxicity of the RS virus-infected cells. The prozone phenomenon was observed in approximately half of the sera examined with IgG antibody titres of 1/1000 or greater. It is possible that the prozone phenomenon resulted from the use of heat-inactivated sera in the ADCC assay, as heating is known to aggregate Ig molecules. It may be possible to eliminate the prozone by washing the cells in order to remove the IgG aggregates.

There is now evidence that different effector cells can mediate ADCC, depending on the kind of target cell used in the assay system. The effector cells mediating ADCC to cells are non-adherent and non-phagocytic, in contrast to the effector cells for antibody-coated erythrocytes which adhere to glass surfaces and are phagocytic (MacDonald *et al.*, 1975; Pollack *et al.*, 1976). In the RS virus system effector cell populations depleted of adherent cells mediated maximal cytotoxicity, whereas those enriched in adherent

cells were unable to mediate ADCC. Thus, the effector cells involved in the RS virus ADCC assay are non-adherent to glass surfaces.

The ADCC assay for RS virus was found to be specific for the eight sera and six colostra specimens examined (Tables 2 and 3), in that ADCC was only detected in those specimens with RS virus antibody as detected by membrane fluorescence.

A comparison of the results obtained by ADCC and by membrane fluorescence suggested that both IgA and IgG antibody to RS virus were capable of mediating cytotoxicity. It is hoped that more detailed analysis of the immunoglobulin classes capable of mediating ADCC in the RS virus system may be possible in the future by improvement of the quantitative aspects of the ADCC assay.

In most of the systems investigated, the antibody class responsible for ADCC has been IgG. Recently, however, IgM has been found to be active in an ADCC assay for murine sarcoma virus (Lamon *et al.*, 1975). There have been no reports, so far, of IgA being able to mediate ADCC.

In the absence of specific antisera, there was a variation in the release of ^{51}Cr from RS virus infected target cells exposed to effector cells at a ratio of 40:1 (Tables 2 and 3). The amount of isotope released was directly proportional to the effector–target cell ratio. The non-specificity of this cytotoxicity was shown by the similar release for both virus-infected and uninfected target cells obtained for each experiment. This non-specific release was also independent of the effector cell donor. The mechanism involved is unknown, although a similar situation has been reported by Shore *et al.* (1976) in the ADCC assay for herpes virus. It could be speculated that the effector cells may be cytotoxic to either cellular histocompatibility antigens or an unknown virus or mycoplasma indigenous in the HEp 2 cells.

The *in vivo* implication for ADCC of RS virus infected cells is difficult to ascertain. Antibody is present in the sera of infants with severe RS virus respiratory infections and has the ability to mediate *in vitro* ADCC. It is hoped that future studies may determine whether effector cells are present in the peripheral blood of these infants and, if so, whether they are capable of taking part in ADCC or in direct lymphocyte cytotoxicity of RS virus infected cells. The description of ADCC in this virus system is the first stage in the development of a method for the study of cell-mediated immunity to RS virus.

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