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In vitro cell-dependent lysis of respiratory syncytial virus-infected cells mediated by antibody from local respiratory secretions

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SUMMARY

Respiratory syncytial (RS) virus causes a local infection of the respiratory tract which is frequently severe in infants. We report the development in infected infants of antibodies in respiratory secretions capable of mediating *in vitro* destruction of RS virus-infected tissue culture cells in conjunction with non-immune lymphoid cells. The cytotoxic antibody activity was not detectable in nasal secretions from infants hospitalized with respiratory infections where RS virus was not identified. The rise in activity occurred concurrently with recovery from infection and the rise in specific IgG, IgM and IgA antibody levels measured by membrane immunofluorescence assay, but was dissociated from the development of plaque-neutralizing activity. In serum it appears that the cytotoxic antibody belongs to the IgG class as shown by its ability to cross the placenta and by neutralization with specific antiserum. These findings are discussed in relationship to secretory antibody responses in RS virus infection with respect to pathogenesis and recovery.

INTRODUCTION

Local factors are probably important in protection and recovery from disease caused by respiratory syncytial (RS) virus. The role of such factors in protection is inferred from studies in adult volunteers (Mills *et al.*, 1971) and from the inability of serum antibody, placentally derived, to protect small infants against infection and illness. Their role in recovery is inferred from the local nature of the infection and from the appearance of specific antibody in respiratory secretions at the time of recovery from illness and coincident with disappearance of virus (McIntosh *et al.*, 1978). The function of this antibody is, however, not explained. *In vitro*, the antibody, demonstrable by immunofluorescence, appears to lack neutralizing activity.

In this study evidence is presented indicating that some local antibody is able to participate in the cell-dependent lysis of virus-infected cells. The antibody-dependent cell-mediated cytotoxicity (ADCC) system as a mechanism of *in vivo* recovery from RS virus infection is discussed.

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MATERIALS AND METHODS

Patients studied and treatment of specimens. Serial nasopharyngeal secretions (NPS) were obtained from infants with RS virus infections as part of a study of antibody in nasal secretions previously reported (McIntosh, McQuillin & Gardner, 1979). A total of 30 specimens were examined. They were obtained during hospitalization from 11 infants aged between 7 weeks and 7 months. Seven of the patients had bronchiolitis, one pneumonia and three mild respiratory illness. RS virus in NPS was detected by the technique of fluorescent antibody staining (Gardner & McQuillin, 1974) as part of the routine diagnostic service.

In addition to NPS from RSV-infected infants, eight secretions from infants in whom no virus diagnosis was made were examined in parallel. In all specimens supernatant material was removed following centrifugation to remove cells and was frozen at −40°C. Since follow-up secretions were not obtained from patients in whom no virus diagnosis was made, stored supernatants from the secretions of six infants where serial specimens had been taken following virus diagnosis were also used. Two of these infants had been infected with parainfluenza virus type 2, two with parainfluenza virus type 3, one with adenovirus type 1 and one with rhinovirus.

All stored secretions were thawed and diluted in an equal volume of phosphate-buffered saline, shaken for 1 hr at 4°C with glass beads and centrifuged at 3,500 r.p.m. for 2 hr. The cell-free supernatants were stored at −40°C before testing in the cytotoxicity and fluorescent antibody tests.

None of the secretions examined had grossly detectable blood, and measurement of total immunoglobulins indicated that IgA was always the predominant immunoglobulin.

Paired maternal and cord sera were obtained from women admitted to the Princess Mary Maternity Hospital in Newcastle upon Tyne (Downham *et al.*, 1976) and stored at −20°C before use.

Antibody-dependent cell-mediated cytotoxicy (ADCC) assay. The system employed is fully described by Scott *et al.* (1977) and utilizes ^{51}Cr-release from HEp-2 target cells infected with Long strain RS virus. NPS specimens were added to the assay at a final dilution of 1 in 4. Cord and corresponding maternal sera were diluted to 1 in 500 before addition to the ADCC assay, this having previously been found to be a limiting dilution. Effector cells were obtained from healthy adult volunteers, prepared by centrifugation on Ficoll–Hypaque, and added to the assay at an effector to target cell ratio of 80:1.

Controls for lysis of non-infected target cells, for lysis in the absence of secretion or serum, and in the absence of effector cells as well as for spontaneous leakage of ^{51}Cr were included in each experiment. Known positive reference sera were also included on each plate.

Per cent specific ^{51}Cr-release due to ADCC was calculated from the following formula:

$$\text{Per cent specific } ^{51}\text{Cr-release} = \left[\frac{C_T - C_S}{C_M - C_S} - \frac{C_L - C_S}{C_M - C_S}\right] \times 100,$$

where C_T = counts per minute (c.p.m.) from infected cells + antibody + effectors; C_S = spontaneous release; C_M = maximum release (decon.); C_L = release in presence of lymphocytes alone.

All determinations were carried out in triplicate and mean counts calculated. Per cent specific ^{51}Cr-release from non-infected cells was not found to exceed that from infected cells in any of the determinations.

A variable direct lysis of RS virus-infected target cells in the absence of serum or NPS was seen; however, this was compensated for in the formula for the calculation of per cent specific release of ^{51}Cr. No lysis in the absence of mononuclear cells was seen. The proportion of virus-infected cells in each experiment was found to be between 40 and 50% as assessed by membrane fluorescence using a hyperimmune rabbit RS virus serum.

As a control for the virus specificity of the ADCC reaction, infected and uninfected cells were mixed together in varying proportions before reaction with a positive reference serum. A linear relationship between the per cent specific ^{51}Cr-release and the percentage of RS virus-infected cells as determined by membrane immunofluorescence was seen.

Neutralization of ADCC activity in sera was carried out using anti-human immunoglobulin class sera raised in sheep (Wellcome Reagents).

Antiviral antibody determinations. Antiviral antibody levels in NPS were determined as part of a study of nasal antibody levels in RS virus infections and details of the method have previously been reported (McIntosh *et al.*, 1979). The technique of membrane immunofluorescence on suspended infected tissue culture cells was employed. The Long strain of RSV was used throughout. Antibody-coated cells were labelled with fluorescein-conjugated anti-human IgG, IgA or IgM sera raised in sheep (Wellcome Reagents). Total IgA and IgG levels were measured on Oxford low-level plates (Searle Diagnostics).

Plaque reduction assay. Neutralizing activity in NPS was measured by 60% plaque reduction in HEp-2 cells infected with the Long strain of RSV. NPS were inactivated at 56°C for 30 min before testing, and no source of complement was added to the test. The protocol followed was that of McIntosh *et al.* (1978). A standard serum was included in each test, and titres were estimated by probit analysis.

RESULTS

All acute-phase nasal secretions from RSV-infected infants were taken within 3 days of hospital admission and showed virus-infected cells by immunofluorescence. In Fig. 1 ADCC in these specimens is compared with that in specimens obtained as late as possible following initial diagnosis (mean of 7 days post-admission). In six of the convalescent specimens RS virus-infected cells were no longer detectable and a rise in ADCC value was seen. In the other five specimens RS virus-infected cells were still detectable, and three showed a rise in ADCC value. Of six other pairs of NPS specimens taken from infants with infections by viruses other than RS virus, none showed detectable ADCC activity to RS virus-infected cells in either the acute or convalescent phases. This difference in response is statistically significant ($P = 0{\cdot}004$; Fisher's exact probability test). ADCC activity was also undetectable in eight acute NPS specimens in which viruses were not identified.

The development of ADCC activity, RS virus-specific antibodies and plaque reduction neutralizing activity with respect to day of hospitalization are shown in Figs 2 and 3. The charts are constructed from the composite data obtained from the analysis of 30 NPS specimens taken from the 11 infants with RS virus infections. Adjustment of the antibody and plaque-reducing activities to a standard total antibody concentration (mg% total IgA or IgG) does not alter the overall pattern seen, and as shown in Fig. 2d, the mean total IgA and IgG levels do not vary significantly. In some

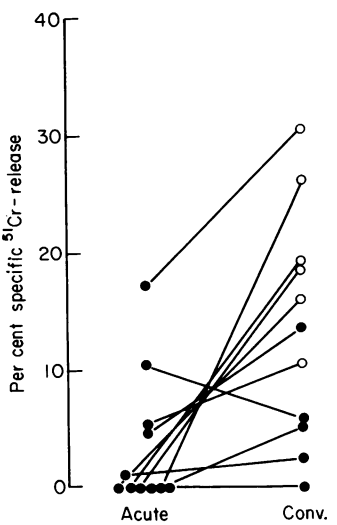

Fig. 1. Acute ⩽ 3 days of hospitalization) and convalescent-phase antibody levels in nasopharyngeal secretions from 11 infants with respiratory syncytial (RS) virus infections, detected in the antibody-dependent cell-mediated cytotoxicity (ADCC) reaction. (●) Specimens where RS virus-infected cells were detectable in secretion; (○) specimens where no virus-infected cells were detected.

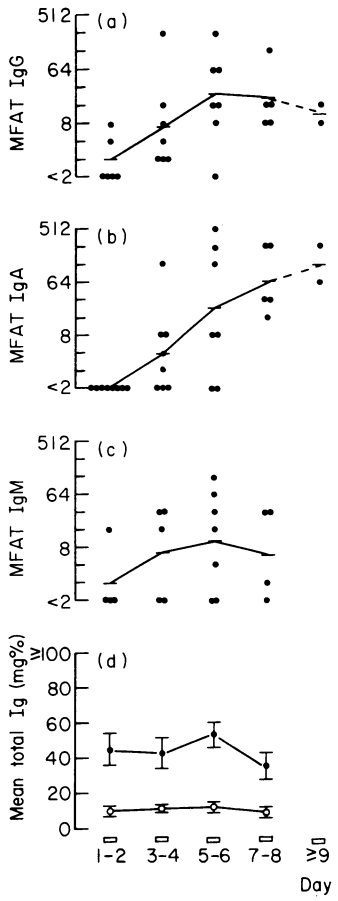

Fig. 2

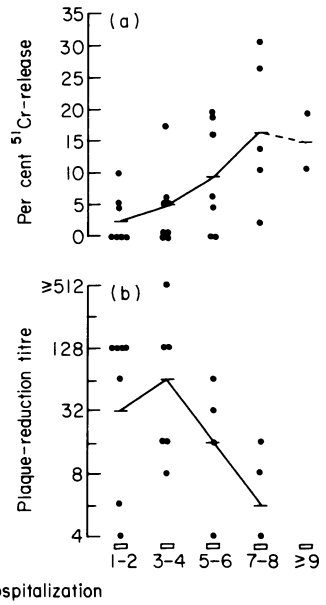

Fig. 3

Fig. 2. Development of isotype-specific antibody in nasopharyngeal secretions from infants with respiratory syncytial virus infections. (a, b & c) RS virus-specific antibody titres measured in the membrane fluorescent antibody technique (MFAT). Total antibody levels measured by immunodiffusion are shown in (d): (●——●) IgA; (○——○) IgG. Bars indicate geometric mean titres.

Fig. 3. Development of antibody able to mediate cell-mediated cytotoxicity (a) and of respiratory syncytial (RS) virus plaque-reducing activity (b) in nasopharyngeal secretions from infants with RS virus infections. The infants and secretions are the same as those shown in Fig. 2.

instances insufficient material was available for determination in all tests; furthermore, in seven specimens taken from two patients, positive IgM fluorescent staining was obtained with control cells, and thus determination of IgM titres in these specimens was not possible. In no instance was non-specific staining observed with IgG or IgA fluorescein conjugates. A positive correlation was obtained between ADCC and either IgG or IgA antibody titres ($r=0{\cdot}56$; $P<0{\cdot}01$ and $r=0{\cdot}64$; $P<0{\cdot}001$ respectively by product moment correlation). However, IgG and IgA titres showed co-correlation ($r=0{\cdot}58$; $P<0{\cdot}01$). No correlation, however, was obtained between ADCC and plaque reduction neutralizing titres ($r=0{\cdot}27$). Owing to low numbers, data for IgM antibody titres were not analysed.

Insufficient material was available to characterize the class of antibody responsible for cell lysis in secretions. However, serum specimens originally used in the standardization of the test were available as well as cord and corresponding maternal sera. From these specimens evidence was obtained that, at least systemically, ADCC activity resides in the IgG fraction. The results of

Table 1. Respiratory syncytial virus antibody titres determined by membrane immunofluorescence in maternal–cord serum pairs compared to per cent specific release of ^{51}Cr measured in the antibody-dependent cell-mediated cytotoxicity (ADCC) reaction at a dilution of 1 in 500

Specimen number		Reciprocal titre IgG	IgM	IgA	ADCC (% specific ^{51}Cr-release)
9419	(M)	64	<4	4	25
9421	(C)	256	<4	<4	34
9344	(M)	32	<4	4	16
9346	(C)	64	<4	<4	20
9289	(M)	64	<4	8	25
9291	(C)	256	<4	<4	40
9749	(M)	32	<4	8	10
9751	(C)	128	<4	<4	19
9240	(M)	128	<4	32	19
9242	(C)	128	<4	<4	24
9640	(M)	128	<4	32	18
9642	(C)	256	<4	<4	21

M = maternal, C = cord.

antibody determinations by membrane immunofluorescence and ADCC assays in six pairs of maternal and cord sera are shown in Table 1. IgG titres to RS virus were frequently higher in the cord sera than in the corresponding maternal sera. Although IgM was not detectable in any of the maternal bloods, IgA was invariably present, and total exclusion of this class of antibody from cord blood was seen. ADCC activity measured at a 1/500 dilution of the sera was found to be transferred transplacentally with a corresponding increase in activity seen in cord specimens.

Further evidence for IgG as the antibody class involved in the ADCC reactivity in serum was

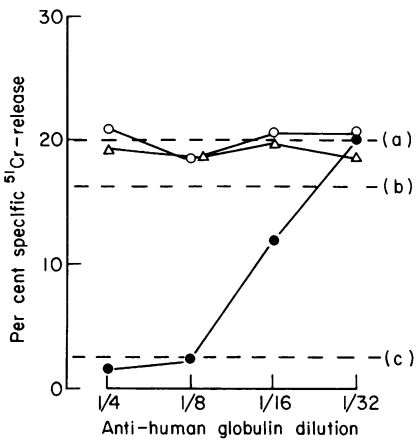

Fig. 4. Neutralization of serum antibody able to mediate respiratory syncytial virus-specific antibody-dependent cell-mediated cytotoxicity (ADCC). (●——●) Anti-human IgG, (○——○) anti-human IgA, (△——△) anti-human IgM. (a) Level of ADCC antibody in untreated serum in the presence of normal lymphocytes. (b) Level of ADCC antibody in untreated serum detectable following pre treatment of lymphocytes with a mixture of serum and anti-human IgG. (c) Per cent specific release of ^{51}Cr in the absence of serum. Each point represents the mean of three experiments.

obtained in neutralization experiments (Fig. 4). Complete abrogation of ADCC activity was seen when sera known to contain antibody of all three isotypes were incubated with anti-human IgG prior to addition to the target cells in the ADCC assay. Incubation of sera with anti-human IgA and anti-human IgM preparations had no effect. It was not found to be possible to pre-arm lymphocytes with serum. Incubation of lymphocytes with a mixture of serum and anti-human IgG before addition to sensitized target cells resulted in a small reduction in ADCC activity, indicating that neutralization was not due, at least in the main, to Fc blockade of effector cells by immune complexes under the conditions employed in the assay system.

DISCUSSION

In this study it has been shown that antibody able to participate in the *in vitro* cell-dependent lysis of RS virus-infected tissue culture cells is detectable in respiratory secretions from infants recovering from RS virus infections. The lack of detectable ADCC antibody in secretions taken from infants with other virus infections suggests that the response seen in RS virus-infected patients was specifically induced by the virus infection and was not a result of passive exudation of serum antibodies resulting from localized virus-induced damage.

The development of antibodies of the IgG, IgA, and IgM isotypes specific to RS virus in local secretions, was found to mirror closely that of ADCC-sensitizing antibody, and thus it was not possible to correlate differentially antibody class with ADCC activity; however, as shown in Fig. 3, ADCC activity was detectable in several specimens taken on days 1 or 2 of hospitalization. In no case was cell-free IgA detectable at this stage. This suggests that the ADCC activity was due either to IgG or IgM, or both. Alternatively, it is possible that early IgA levels are below the sensitivity of the membrane fluorescence antibody assay but are still detectable by ADCC. By way of contrast, RS virus plaque-reducing activity is present in early secretions and shows a marked decline whilst ADCC and specific antibody levels are still rising.

Characterization of the ADCC activity in NPS was not possible owing to the limited quantity of material available; it was, however, possible to show that ADCC-sensitizing antibody may be transferred transplacentally. In two maternal sera, where a relatively high IgA titre of 1 in 32 was measured, the complete loss of this component in the corresponding cord sera did not result in a reduction of ADCC activity. Also, IgG titres were usually found to be higher in cord sera than in the corresponding maternal specimen; the equivalent increase in ADCC activity suggested that the ADCC activity was IgG-mediated. A similar transplacental concentration effect has been reported by Heijtink *et al.* (1976) for complement-fixing antibody to RS virus. Further evidence that the ADCC activity in serum resides in the IgG fraction was obtained in neutralization experiments where a proportionate loss of ADCC activity was observed upon incubation of sera with dilutions of an anti-human IgG serum. No similar effect was seen with anti-human IgM and IgA sera. These findings support those recently reported by Meguro, Kervina & Wright (1979) who showed that serum ADCC activity to RS virus-infected cells was associated with an IgG fraction prepared on sucrose gradients and could be absorbed with staphylococcal protein. A

Clearly, further work on pooled respiratory secretions is needed to confirm the nature of the local ADCC-sensitizing antibody. The ADCC reaction offers an interesting *in vitro* model for a mechanism that may act locally in the recovery process. Consideration of the growth characteristics of RS virus, at least *in vitro*, indicates the possible importance of mechanisms such as ADCC in limitation of infection. Levine & Hamilton (1967, 1969) have shown RS virus to be relatively cell-associated. The virus may spread by cell-to-cell fusion through the characteristic generation of syncytia and is released from the cell by budding through the cell membrane where virus-specified antigens appear. Thus a limiting stage of the infection process is the requirement for membrane budding, and host defence mechanisms directed against virally altered cells may be expected to be important in the limitation of infection and in recovery. As reported by Gardner & McQuillin (1978) and by McIntosh *et al.* (1979) RS virus-infected cells coated with immunoglobulin are seen in NPS from infants with RS virus infections. It is not known whether these cells are coated *in situ* within the respiratory tract and are subsequently sloughed off, or whether cells are released by direct

viral damage and mechanical action and subsequently become coated with antibody, but it seems likely that a complex dynamic balance exists. That infants possess systemic effector cells for ADCC has been shown by Meguro *et al.* (1979) and further work is needed to test the local functional cellular adequacy of infants.

McIntosh *et al.* (1979) observed that whilst the appearance of IgA-coated cells precedes the appearance of cell-free IgA in respiratory secretions from infants with RS virus infections, cell-free IgG is frequently detected in the absence of its cell-bound counterpart. It may be that cells coated with IgG are susceptible to lysis *in vivo*. The peribronchiolar infiltration of lymphocytes seen at post-mortem in bronchiolitis (Aherne *et al.*, 1970) indicates the availability of lymphocytes in the lungs and their association with sites of tissue damage. It seems probable that ADCC could operate in this situation.

That the neonate receives transplacental ADCC-sensitizing antibody to RS virus-infected cells has been shown both in this study and in that of Meguro *et al.* (1979). These latter workers found that the disappearance of ADCC antibody following primary infection of infants was much more rapid than that of neutralizing activity. Placentally transferred antibody, on the other hand, decayed at the same rate when measured by ADCC or neutralization. Thus, ADCC antibody may represent a short-lived subclass of immunoglobulin which is generated locally and systemically in response to infection. The failure to detect ADCC antibody in secretions from infants without RS virus infection suggests that prior RS virus infection or transfer of immunity either through placental IgG or breast milk does not induce persistent detectable activity on the mucosal surface.

Thus, the ADCC system provides a method for the assessment of functional antibody *in vitro*, and it is envisaged that the reaction could be one of several post-infective processes operating in the respiratory tract during RS virus infection. T cell and natural killer (NK) activities may also be important in the removal of virus-infected cells. The demonstration that RS virus is a poor inducer of interferon in the respiratory tract (Hall *et al.*, 1978; McIntosh, 1978) and that interferon is a potent inducer of NK activity *in vitro*, may mean that antibody-directed mechanisms are particularly important in disease due to this virus.

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