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The local antibody response to R.S. virus infection in the respiratory tract

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SUMMARY

Nasopharyngeal secretions were taken during the acute phase of illness from 66 infants and children admitted to hospital with lower respiratory tract infections. Second secretions were taken, after an interval of 7 days, from 33 of these patients. A significant increase in neutralizing activity to R.S. virus was demonstrated in the nasopharyngeal secretions of patients in response to severe R.S. virus infection. Seventeen out of 25 patients (68%) with R.S. virus infections developed a rise in secretory neutralizing titre, compared with only 1 out of 8 patients (13%) with respiratory infections not involving R.S. virus.

A high titre of secretory neutralizing activity was found more often in the acute phase of illness in patients with R.S. virus infections, especially bronchiolitis, than in patients with respiratory infections not involving R.S. virus. Fifteen out of 34 patients (44%) with R.S. virus bronchiolitis were found to possess a neutralizing titre of 1/4 or more in their first secretions, compared with 4 out of 12 patients (33%) with R.S. virus infections other than bronchiolitis and 3 out of 20 patients (15%) with respiratory infections not involving R.S. virus.

A quantitative analysis of the immunoglobulins present in the secretions indicated that IgA was the only immunoglobulin consistently present at a detectable concentration. The geometric mean values of IgA, IgM and IgG in the secretions examined were found to be 22·3 ,4·3 and 5·3 mg./100 ml. respectively.

The neutralizing activity against R.S. virus, present in the secretions, was shown to be due to specific IgA antibody. This was accomplished by removing the neutralizing activity in two secretions by absorption with anti-IgA serum.

INTRODUCTION

Respiratory syncytial (R.S.) virus has been shown to be the chief viral pathogen in respiratory infections of infancy and the one most frequently associated with bronchiolitis (Chanock *et al.* 1961; Gardner, 1968). Severe infection with this virus occurs predominantly in infants between 2 and 6 months of age. The moderate to high levels of maternally derived serum antibody, present in the sera of infants of this age, does not appear to protect against infection. In spite of attempts to produce an inactivated vaccine (Kapikian *et al.* 1969; Kim *et al.* 1969*a*) the problem of prevention of infection with this virus remains unsolved. In fact these studies

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with inactivated vaccine have shown that those who have been given vaccine fare worse than those without, on natural infection with R.S. virus, despite the production of humoral antibody.

Many workers' attention is now turning to studies on the importance of local secretory antibody in resistance to infection by respiratory viruses; these include studies on influenza A_2 (Alford, Rossen, Butler & Kasel, 1967), rhinovirus type 13 (Perkins *et al.* 1969) and parainfluenza type 1 (Smith, Purcell, Bellanti & Chanock, 1966).

The present study was initiated to investigate the role of local antibodies in R.S. virus infection of infancy. In a recent study of experimental R.S. virus infection of adult volunteers, Mills, Van Kirk, Wright & Chanock (1971) reported that resistance to infection with R.S. virus was correlated with high titres of nasal wash antibody, but not with the titre of serum antibody. It is not known, as yet, whether the same is true for natural R.S. virus infection of infants and young children. However, the development of local neutralizing response to R.S. virus in nasal secretions of infants with natural infection has been demonstrated by workers both in the U.S.A. (Kim *et al.* 1969*b*) and in this country (Scott & Gardner, 1970).

An important aim of the present study was to confirm and expand the previous results and to correlate the different categories of acute respiratory illness with local antibody response. This may be of particular importance with respect to the various views expressed on the pathogenesis of R.S. virus (Chanock *et al.* 1970; Gardner, McQuillin & Court, 1970).

Another aim was to study the immunoglobulin content of nasopharyngeal secretions of infants with severe respiratory infection. A number of investigations have shown that the predominant immunoglobulin in adult nasal secretions is IgA (Remington, Vosti, Lietze & Zimmerman, 1964; Rossen, Schade, Butler & Kasel, 1966). Moreover, antibody activity in adult nasal secretions, following various rhinovirus, influenza and parainfluenza infections, has been predominantly in the IgA fraction (Cate *et al.* 1966; Alford *et al.* 1967; Smith, Bellanti & Chanock, 1967; Perkins *et al.* 1969). There have been few parallel studies for the immunoglobulins of nasal secretions of infants and young children (Haworth & Dilling, 1966; Cohen, Goldberg & London, 1970). Cohen, Goldberg and London reported that the predominant immunoglobulin in the nasal secretions of 34 healthy infants was IgG, whereas IgA was found to be predominant in the nasal secretions of 19 infants with acute respiratory infections.

A further aim of the present study was to obtain a quantitative estimate of the concentrations of immunoglobulins in the secretions of infants with severe respiratory infections. We also wished to determine whether the neutralizing activity in the secretions was due to antibody and, if so, which particular class of immunoglobulins was responsible.

MATERIALS AND METHODS

Patients

The patients included in this study were children between 2 weeks and 2 years of age, who were admitted to hospital with acute lower respiratory tract infections

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The categories of clinical illness used have been previously defined (Gardner *et al.* 1960). A nasopharyngeal secretion was obtained from the patient within 24 hr. of admission to hospital; the onset of illness was rarely more than 48 hr. before the first specimens were taken. Whenever possible, a second secretion was obtained from the patient after an interval of approximately 7 days. The method of collection and preparation of secretions has been fully described elsewhere (Sturdy, McQuillin & Gardner, 1969; Scott & Gardner, 1970).

Estimation of neutralizing activity

The neutralizing activity in nasopharyngeal secretions was measured by a modification of the R.S. virus plaque reduction technique, first described by Coates, Alling & Chanock in 1966 and described in detail elsewhere (Scott & Gardner, 1970). The protein content of the secretions was measured by a microtechnique based on the original method of Lowry, Rosebrough, Farr & Randall (1951), and the neutralizing titres of the secretions were adjusted to a protein level of 10 mg./ml.

Estimation of immunoglobulin content

The concentrations of IgA, IgM and IgG in the secretions were determined quantitatively by radial immunodiffusion in agar (Mancini, Carbonara & Heremans, 1965). The assay of each immunoglobulin was carried out in Hyland Immunoplates. It must be emphasized that sera, and not secretions, of known immunoglobulin content were used as standards in all tests.

Removal of IgA by precipitation with specific antiserum

The method used for the removal of IgA from the secretions was based on the technique employed by Fireman, Vannier & Goodman (1963). Antiserum against human IgA produced in goats (Hyland Laboratories) was added to an equal volume of secretion. The mixture was incubated at 37° C. for 2 hr., and subsequently at 4° C., for 18 hr.; it was then centrifuged at 2000 rev./min. for 2 hr., after which the supernatants were tested for the presence of IgA.

RESULTS

Antibody response in nasopharyngeal secretions

Nasopharyngeal secretions taken from 66 patients, within 24 hr. of admission to hospital, were examined for the presence of neutralizing activity to R.S. virus. Second secretions were taken approximately 7 days after admission from 33 of these patients, of whom 19 had R.S. virus bronchiolitis, 6 had R.S. virus infections *other than bronchiolitis (bronchitis and pneumonia), and 8 had acute respiratory* infections (bronchiolitis, bronchitis and pneumonia) not associated with R.S. virus (Table 1). Eleven of the 19 patients (58 %) with R.S. virus bronchiolitis developed a rise in titre of neutralizing activity in their second secretions compared with their first (both adjusted to 10 mg./ml. protein). All 6 patients with R.S. virus infections other than bronchiolitis developed a rise in titre. A rise in nasal secretory neutralizing activity was detected, therefore, in 17 out of 25 patients (68 %)

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Table 1. *Neutralizing activity to R.S. virus in paired nasopharyngeal secretions of infants and children with severe respiratory infection*

Clinical category	No. in group	Average age (weeks)	No. of rises in titre of neutralizing activity to R.S. virus	No. of 4-fold rises in titre of neutralizing activity to R.S. virus
R.S. virus bronchiolitis	19	14	11 (58%)	7 (37%)
R.S. virus infection other than bronchiolitis	6	23	6 (100%)	2 (33%)
Respiratory infections not involving R.S. virus	8	25	1 (13%)	0

Table 2. *Neutralising activity to R.S. virus in first nasopharyngeal secretions of infants and children with severe respiratory infection*

Clinical category	No. in group	Average age (weeks)	No. of secretions with neutralizing titres of 1/4 or greater
R.S. virus bronchiolitis	34	15	15 (44%)
R.S. virus infections other than bronchiolitis	12	23	4 (33%)
Respiratory infections not involving R.S. virus	20	36	3 (15%)

with R.S. virus infections, 9 of these rises were 4-fold or greater, and a further 4 were 3-fold. In the group of 8 patients with respiratory infections not associated with R.S. virus, 1 patient (13%) developed a rise in neutralizing activity, which was 2-fold.

Antibody in first secretions

A neutralizing titre of 1/4 or greater was detected in the first secretions of 15 out of 34 patients (44%) admitted with R.S. virus bronchiolitis (Table 2). This compared with 4 out of 12 patients (33%) in the group of patients with R.S. virus infections other than bronchiolitis (bronchitis, pneumonia and croup). Only 3 of the 20 patients (15%) with respiratory infections not associated with R.S. virus (bronchiolitis, bronchitis, pneumonia and croup) were found to possess a neutralizing titre of 1/4 or greater in their first secretions. The differences between these three groups of patients were further emphasized by comparing the geometric mean reciprocal neutralizing titres in their first secretions. These were found to be 4·1, 2·8 and 2·7, respectively. The average ages of the three groups were 15, 23 and 36 weeks (Table 2), which makes these findings even more surprising; it might be assumed that older age groups have had an increased chance of exposure to R.S. virus antigen.

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Table 3. *Geometric mean immunoglobulin content of nasopharyngeal secretions of infants and children with severe respiratory infections*

	Geometric mean immunoglobulin content (mg./100 ml.)					
	First secretion			Second secretion		
Clinical category	IgA	IgM	IgG	IgA	IgM	IgG
R.S. virus bronchiolitis	23·1	3·1	4·5	27·4	4·2	3·4
R.S. virus infections other than bronchiolitis	19·7	4·1	7·7	25·7	3·8	5·5
Respiratory infections not involving R.S. virus	27·7	7·9	10·7	9·3	4·7	2·6
All respiratory infections	23·2	4·4	6·5	20·6	4·2	3·5

Table 4. *Relationship of age to the geometric mean concentrations of immunoglobulins in the first nasopharyngeal secretions of infants and children with severe respiratory infections*

Age (months)	No. in group	Geometric mean concentrations of immunoglobulins (mg./100 ml.)		
		IgA	IgM	IgG
0–2	17	19·0	4·5	6·2
2–4	19	27·3	4·2	4·0
4–6	4	15·1	5·4	4·5
6–12	19	23·8	4·9	10·7
> 12	5	30·8	4·3	13·1

Standardization by IgA

In the present study, protein content was used to standardize the neutralizing titres of the secretions; others (Kim *et al.* 1969*b*) have used IgA content (10 mg./100 ml.). When IgA content (20 mg./100 ml.) was used for standardization in the present study the results were found to be similar to those obtained using protein standardization. Of patients with R.S. virus infections 73 %, compared with 68 %, developed a rise in secretory neutralizing activity in response to infection. After IgA adjustment, 30 % of patients with R.S. virus bronchiolitis, 9 % of patients with R.S. virus infections other than bronchiolitis, and 10 % of patients with respiratory infections not associated with R.S. virus, were found to possess a neutralizing titre of 1/4 or greater in their first secretions. The corresponding figures obtained for these three categories using protein standardization were 44 %, 33 %, and 15 %.

Immunoglobin content

The 66 first and 33 second secretions, previously examined for neutralizing activity to R.S. virus, were also quantitatively examined by radial immunodiffusion for concentration of IgA, IgM and IgG. The geometric mean values of immunoglobulins in the first secretions were 23·2 mg. IgA/100 ml., 4·4 mg. IgM/100 ml. and 6·5 mg. IgG/100 ml. (Table 3). Values of 20·6, 4·2 and 3·5 mg./100 ml. were obtained for the means of the three immunoglobulins in the second secretions (Table 3). No correlation was found between the clinical categories of illness,

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whether caused by R.S. virus or not, and the concentration of immunoglobulins in either first or second secretions. The only exception to this was the abnormally low value obtained for the mean IgA concentration (9·3 mg./100 ml.) in the second secretions of patients with respiratory infections not associated with R.S. virus. The difference between IgA in first compared with second secretions was not considered to be significant, owing to the small number of readings obtained in this category. When the three groups of patients (Table 3) were considered separately, however, the geometric mean concentration of IgA was found to be slightly higher in second compared with first secretions, whereas the reverse was true for IgG.

IgA was detectable in 94·8% of secretions, compared with 58·5% for IgM and 65·5% for IgG, which further emphasized the predominance of IgA in secretions. Moreover, the range of values obtained for IgA was 0–64 mg./100 ml. (S.D. 1·49) compared with 0–47 mg. IgM/100 ml. (S.D. 3·77) and 0–188 mg. IgG/100 ml. (S.D. 4·54). The geometric mean concentrations of IgA and IgG, but not IgM, in the first secretions rose gradually with age (Table 4).

Neutralizing activity and immunoglobulins

Two first secretions, with known neutralizing activity, were absorbed with anti-IgA serum in order to establish whether the neutralizing activity was due to specific IgA antibody. The quantity of secretions available permitted absorption of only one immunoglobulin. IgA absorption was chosen, because previous immunoglobulin analysis had shown that IgA was the predominant immunoglobulin in the secretions. Each secretion was divided into two equal parts, one of which was treated with anti-IgA serum and the other with phosphate buffered saline (unabsorbed control): both were otherwise treated in exactly the same way. After absorption, both parts were tested for the presence of neutralizing activity to R.S. virus. Neutralizing titres of 1/16 and 1/4 were detected in the unabsorbed secretions, compared with titres of less than 1/4 in the two corresponding absorbed secretions. This indicates that the neutralizing activity to R.S. virus in the two secretions was due to specific IgA antibody.

DISCUSSION

In view of the ability of R.S. virus to cause severe illness in infancy in the presence of either natural or actively produced humoral antibody a rational approach to immune prophylaxis against infection would appear to be by way of local immunity. It has been shown in a study of experimental R.S. virus infection of adults (Mills *et al.* 1971), that resistance to infection is more closely related to high titres of nasal wash antibody than to serum antibody. The development of R.S. virus neutralizing activity in nasal secretions has been demonstrated in 11 out of 17 infants and children (65%) with natural R.S. virus infection of the lower respiratory tract (Kim *et al.* 1969*b*). Recent studies have shown that it is possible to induce a local immune response to R.S. virus infection in infants by means of a live attenuated vaccine (Kim *et al.* 1971). A 3-fold or greater rise in nasal secretory neutralizing activity to R.S. virus was induced in 15 out of 39 infants and children (38%).

The present study has also demonstrated a specific local immune response to

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natural R.S. virus infection of infants and young children (Table 1). Seventeen out of 25 patients (68%) with R.S. virus infections developed a rise in secretory neutralizing titre to R.S. virus, compared with only 1 out of 8 patients (13%) with respiratory infections not associated with R.S. virus ($\chi^2 = 7{\cdot}53$; $0{\cdot}01 > P > 0{\cdot}005$). Nine of these 17 rises in titre were 4-fold or greater; a further 4 of the 17 rises were 3-fold. The only rise in titre in the group of patients with respiratory infections not associated with R.S. virus was a 2-fold rise. The results obtained for the development of secretory response were comparable with those of Kim and her colleagues (1969*b*). This was surprising as the interval between secretions in that particular study was 3 weeks, compared with approximately 7 days in the present study.

An important observation made in the present study was that neutralizing activity is more prevalent, during the acute phase of illness, in patients with R.S. virus infections, especially bronchiolitis, than in the patients with respiratory infections not involving R.S. virus (Table 2). There is a significant difference between the figures for patients with R.S. virus bronchiolitis compared with patients with respiratory infections not associated with R.S. virus ($\chi^2 = 4{\cdot}80$; $0{\cdot}05 > P > 0{\cdot}025$). The difference between these two groups was further emphasized by the fact that the average age of the second group was more than twice that of the R.S. virus bronchiolitis group (Table 2). The R.S. virus bronchiolitis group also showed the highest geometric mean reciprocal titres for first secretions.

The presence of neutralizing activity in the first secretions of patients with R.S. virus infections, especially bronchiolitis, could be a manifestation of the presence of non-specific viral inhibitors, the rapid development of nasal secretory antibody, or prior infection with R.S. virus. The first explanation would seem unlikely, as the results have shown a higher incidence of neutralizing activity in the first secretions of patients with R.S. virus bronchiolitis, than in patients with R.S. virus infections other than bronchiolitis and with respiratory infections not associated with R.S. virus. Furthermore, the results of the absorption experiments have indicated that the secretory neutralizing activity is due to IgA antibody. Rapid development of antibody in secretions can be proved only after a longitudinal study of antibody in secretions, collected before exposure to R.S. virus and frequently thereafter. This was not, however, within the scope of the present investigation. The acute onset of R.S. virus illness did not support the hypothesis of a rapid primary antibody response. It is possible, therefore, that these results are an indication of prior infection with R.S. virus. A hypothesis of prior infection would tend to support the theory of a type 1 allergic reaction (Gell & Coombs, 1968), which has been postulated to explain the pathogenesis of R.S. virus bronchiolitis in infants (Gardner *et al.* 1970).

The results of the immunoglobulin analysis indicated that IgA was the only immunoglobulin consistently present in the nasopharyngeal secretions. The geometric mean concentrations of IgA, IgM and IgG in the 99 secretions (first and second) examined were 22·3, 4·3, and 5·3 mg./100 ml. Similar values for the three immunoglobulins were found in both first and second secretions (Table 3). The geometric mean concentrations of IgA and IgG, but not IgM, tended to gradually increase with age (Table 4).

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The IgA concentrations found in the present study were of the same order as those reported in two other studies, in which nasal secretions from infants and children were examined (Haworth & Dilling, 1966; Cohen *et al.* 1970). The same did not apply, however, to the concentrations of IgG in the secretions. Haworth and Dilling reported mean IgA values of 14·5 and 11·7 mg./ 100 ml. in two groups of patients with respiratory infections. Cohen, Goldberg and London showed that the mean concentrations of IgA and IgG in nasal secretions of 34 healthy infants were 22·8 and 55·7 mg./100 ml., compared with 46·6 mg. IgA/100 ml. and 42·5 mg. IgG/100 ml., in the secretions of a group of 19 infants with respiratory infections.

The same technique was used in all three studies for the estimation of immunoglobulin concentrations, although the method of obtaining secretions from patients differed. In addition, secretions of infants and children without respiratory infections were not included in the present study, because the method used for obtaining secretions was impracticable for these patients.

The final part of the present investigation was concerned with determining the nature of the neutralizing activity to R.S. virus detected in the secretions. It is very difficult to explain the results obtained (Tables 1, 2) on the basis of non-specific viral inhibitors, as the secretory neutralizing activity was distributed unevenly among the three groups of patients. It is equally improbable that the neutralizing activity is due to interferon, as the rises in neutralizing titre occurred at a time when antibody and not interferon would be increasing.

The secretory neutralizing activity, therefore, is probably due to specific R.S. virus antibody. In order to test this hypothesis the IgA content of two secretions was removed by absorption with anti-IgA serum and the secretions subsequently examined for neutralizing activity to R.S. virus. The choice of secretions was dependent on the amount available, and in both cases first secretions, with known neutralizing titres, were chosen. It was originally intended that all three immunoglobulins would be absorbed, but this proved impracticable owing to insufficient volume of the secretions. The present study has shown IgA to be the predominant immunoglobulin in the secretions (Table 3), as in a number of other investigations which have indicated that antiviral activity in adult nasal secretions resides in the IgA fraction (Cate *et al.* 1966; Alford *et al.* 1967; Smith *et al.* 1967). Therefore, the absorption studies were for IgA only, and it proved possible to remove the R.S. virus neutralizing activity completely from both secretions by absorption with anti-IgA serum. Further confirmation of this specificity has been provided by fluorescent antibody studies and reported elsewhere (Gardner, McQuillin & Scott, 1973).

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