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Respiratory syncytial virus neutralizing activity in nasopharyngeal secretions

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SUMMARY

Nasopharyngeal secretions were taken during the acute phase of illness from 30 children admitted to hospital with lower respiratory tract infections. The presence of neutralizing activity in secretions taken at the onset of illness was demonstrated in 11 out of 15 patients (73 %) with bronchiolitis caused by R.S. virus, as compared with 4 out of 9 patients (44 %) with R.S. virus infections other than bronchiolitis, and 1 out of 6 without R.S. virus infection. Second secretions were taken 7 days later from 10 of the children with R.S. virus infection. Eight of these paired secretions showed an increase of neutralizing activity against R.S. virus. It is suggested that the neutralizing activity, found in secretions taken at the onset of illness, may be a result of previous infection with R.S. virus.

INTRODUCTION

The possibility that nasal secretions of patients recovering from influenza contain antibodies which inhibit influenza A virus was first suggested by Francis (1940). Recent studies which have involved giving parainfluenza type I (Smith, Purcell, Bellanti & Chanock, 1966) and rhinovirus type 13 (Perkins *et al.* 1969) to volunteers have shown that local secretory antibody is more important than serum antibody in protection against subsequent infections. Since respiratory syncytial (R.S.) virus has many properties in common with myxoviruses, and because of its overwhelming importance in both lower respiratory tract infections and respiratory deaths in childhood (Chanock *et al.* 1961; Aherne *et al.* 1970), it is surprising to find, as yet, few reports of the local production of antibody stimulated by infection with this virus (Kim *et al.* 1969*a*). This investigation was initiated to study local antibody response in nasopharyngeal secretions in children with R.S. virus infections. Another aim of the study was to discover whether neutralizing antibody occurred in nasal secretions of patients with R.S. virus infection and if it did, whether children with R.S. virus infection develop increasing antibody titres as part of a local response.

We also wished to investigate the possibility that a sensitivity reaction may be associated with R.S. virus bronchiolitis, as has been suggested by some workers (Gardner, McQuillin & Court, 1970; Chanock *et al.* 1968). Both type 1 and type 3 allergic reactions (Gell & Coombs, 1968) have been postulated to explain the patho-

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genesis of bronchiolitis, and if antibody could be found in the secretions during the acute phase of an R.S. virus infection, then this would tend to support a type 1 allergic reaction.

MATERIALS AND METHODS

Patients

The patients included in this study were children between 4 weeks and 20 months of age who were admitted to hospital with acute lower respiratory tract infections. The categories of clinical illness used have been previously defined (Gardner *et al.* 1960). In most infants with R.S. virus infection the clinical diagnosis was bronchiolitis. A nasopharyngeal secretion was obtained from the patient within 24 hr. of admission to hospital, and, whenever possible, a second secretion was obtained after an interval of 7 days.

Tissue culture techniques

The growth, maintenance, and overlay media used in the plaque technique described below were prepared in 100 ml. amounts. The growth medium for HEp 2 cells consisted of Eagle's minimum essential medium containing 10 ml. calf serum, 50 mg. penicillin, 25 mg. streptomycin, 12·5 mg. nystatin, and 12·5 mg. neomycin. The maintenance medium for HEp 2 cells consisted of medium 199 containing 2 ml. embryo calf serum, 0·6 ml. 0·25% glutamine, and the same concentration of antibiotics as in the growth medium. The overlay medium consisted of Eagle's minimum essential medium containing 10 ml. heat inactivated embryo calf serum, 0·6 ml. 0·25% glutamine, 0·75 g. methyl cellulose, and the same concentrations of antibiotics as in the growth medium with the exclusion of neomycin.

Collection and preparation of specimens

The method of collection of secretions has been fully described elsewhere (Sturdy, McQuillin & Gardner, 1969). The secretion was partially purified by initial centrifugation at 1000 rev./min. for 10 min. at 4° C. in order to deposit the cells. The supernatant was shaken with glass beads in a Griffith's shaker at 4° C. for 1 hr. and subsequently centrifuged at 3000 rev./min. for 2 hr. at 4° C. The supernatant was separated from the mucus and cell debris, and stored at −20° C. until needed for tests.

Estimation of neutralizing activity

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

HEp 2 cells were seeded in disposable tissue culture plates, 35 mm. in diameter and 10 mm. in depth, at a concentration of 5×10^5 cells per plate, in 3 ml. of

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growth medium. After incubation for 24–30 hr. in a humidified atmosphere of 5 % CO_2 in air, the cells formed a monolayer.

A stock of the Long strain of R.S. virus was grown in HEp 2 cells and stored in 1 ml. ampoules in liquid nitrogen. The TCD 50 of the virus stock, as estimated in HEp 2 tissue culture tubes was $10^{-4.5}$. This corresponded to a titre of 10^6 p.f.u./ml., as estimated by the plaque technique.

The virus suspension was diluted in maintenance medium to give a final concentration of 250 p.f.u./ml. when inoculated on the tissue culture plates of HEp 2 cells. The secretions were inactivated at 56° C. for 30 min. and diluted in maintenance medium, in twofold dilutions starting at 1/4. Equal volumes of virus suspension and diluted secretion were mixed; after 1 hr. at room temperature, 0·2 ml. of each virus–secretion mixture was inoculated on two HEp 2 plate cultures, which were incubated at 37° C. for 2 hr. in a humidified atmosphere containing 5 % CO_2 in air. In addition, two plates containing 0·2 ml. diluent which acted as negative controls, and a virus titration, which consisted of three plates containing 0·2 ml. of an equal volume of virus suspension and diluent, were similarly incubated.

After the virus had been allowed to adsorb onto the cells, they were washed with Hanks's balanced salt solution, and 4 ml. amounts of the overlay medium were added to each plate. The plates were reincubated at 37° C. in a humidified atmosphere of 5 % CO_2 in air. Four days later the cells of one virus titration plate were fixed with 10 % formalin and stained with haematoxylin and eosin. According to the stage of development of the plaques, the cells in the remaining plates were either fixed and stained immediately or reincubated for a further 24 hr. The plaques were counted, using a Vickers Sterimag II microscope (magnification × 20) and the titre of neutralizing activity was calculated as the dilution of the secretions producing 60 % or more reduction in the plaque count.

Owing to the very small volume of secretions usually available, it was necessary to dilute some of the secretions in saline, before the estimation of neutralizing activity and protein content.

The cells in the nasopharyngeal secretions were investigated for the presence of R.S. virus by the fluorescent antibody technique (Gardner & McQuillin, 1968) as part of the routine diagnostic investigation on a patient; secretions were also inoculated on HEp 2, Bristol HeLa, monkey kidney and W.I. 38 cell lines, to confirm the presence of any virus (Sturdy *et al.* 1969).

RESULTS

Nasopharyngeal secretions taken from 30 patients within 24 hr. of admission to hospital were examined (Tables 1 and 2). Second secretions taken 7 days after admission from 10 of these 30 patients, were also examined (Table 2). In this group of 10 patients from whom paired secretions were examined, eight developed a rise in titre of nasal secretory neutralizing activity (adjusted to 10 mg./ml. protein). In four of these eight patients the rise in neutralizing activity was fourfold or greater. Furthermore, the presence of neutralizing activity at a level of

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1/10 or greater in the first secretions of the 10 patients (Table 2), appeared to give some indication of the subsequent response. Two of the three patients with a titre of neutralizing activity of 1/10 or greater in their first secretion did not show any increase in neutralizing activity in their second secretion.

Table 1. *Neutralizing activity in acute nasopharyngeal secretions of children with lower respiratory tract infections*

Patient no.	Diagnosis	Age	Isolation of virus in nasopharyngeal secretion	R.S. virus plaque reduction titre	Protein content (mg./ml.)	R.S. virus plaque reduction titre adjusted to 10 mg./ml.
1	Bronchiolitis	5 months	R.S. virus	1/4	3·2	1/13
2	Bronchiolitis	3 months	R.S. virus	1/4	11·4	1/4
3	Bronchiolitis	4 months	R.S. virus	1/4	4·9	1/8
4	Bronchiolitis	7 weeks	R.S. virus	1/16	1·9	1/84
5	Bronchiolitis	2 months	R.S. virus	1/32	8·1	1/40
6	Bronchiolitis	8 weeks	R.S. virus	1/32	10·0	1/32
7	Bronchiolitis	10 months	R.S. virus	< 1/4*	8·5	1/2
8	Bronchiolitis	7 weeks	R.S. virus	< 1/4	6·2	1/3
9	Bronchiolitis	5 weeks	R.S. virus	1/4	11·3	1/4
10	Bronchiolitis	9 months	Adenovirus	< 1/4	11·6	1/2
11	Bronchiolitis	4 weeks	Negative	< 1/4	17·1	1/1
12	Pneumonia	7 months	R.S. virus	< 1/4	9·2	1/2
13	Pneumonia	4 months	R.S. virus	< 1/4	11·2	1/2
14	Pneumonia	4 months	R.S. virus	< 1/4	6·7	1/3
15	Pneumonia	3 months	Parainfluenza III	< 1/4	9·7	1/2
16	Bronchitis	5 months	R.S. virus	< 1/4	6·4	1/3
17	Bronchitis	11 months	R.S. virus	< 1/4	7·2	1/3
18	Croup	20 months	Negative	< 1/4	5·2	1/4
19	Croup	12 months	Parainfluenza I	1/4	16·9	1/2
20	Croup	4 months	Negative	< 1/4	6·2	1/3

* < 1/4 is considered as 1/2 for adjusting R.S. virus plaque reduction titres to 10 mg./ml. of protein.

In 11 of the 15 patients (73%) admitted with bronchiolitis, and from whom R.S. virus was isolated, a neutralizing titre of 1/4 or greater was found in their first secretion (Tables 1 and 2). However, in the nine patients with illnesses other than bronchiolitis, and from whom R.S. virus was isolated, only four (44%) had a neutralizing titre of 1/4 or greater in their first specimen. Furthermore, only one of the six patients, from whom R.S. virus was not isolated, possessed neutralizing activity at a titre of 1/4 or greater in the first secretion.

The development of a rise in titre of secretory neutralizing activity, or the presence in the secretions of a high neutralizing titre during the acute phase of illness did not appear to be related to age.

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Table 2. *Neutralizing activity in paired nasopharyngeal secretions of children with lower respiratory tract infections*

Naso-pharyngeal secretion	Patient no.	Diagnosis	Age	No. of days between secretion	Virus diagnosis	Reciprocal R.S. virus plaque reduction titre	Protein content (mg./ml.)	Reciprocal of R.S. virus plaque reduction titre adjusted to 10 mg./ml.
First	21	Bronchiolitis	3 months	6	R.S. virus	8	7·8	10
Second					Negative	4	15·5	3
First	22	Bronchiolitis	5 months	7	R.S. virus	< 4*	5·3	4
Second					R.S. virus	8	6·3	13
First	23	Bronchiolitis	10 weeks	7	R.S. virus	4	10·4	4
Second					Negative	32	3·2	100
First	24	Bronchiolitis	10 weeks	7	R.S. virus	< 4	11·7	2
Second					Negative	8	11·9	7
First	25	Bronchiolitis	12 weeks	7	R.S. virus	< 4	1·1	18
Second					R.S. virus	8	6·1	13
First	26	Bronchiolitis	4 months	6	R.S. virus	< 4	16·3	1
Second					R.S. virus	8	6·9	12
First	27	Bronchitis	10 months	4	R.S. virus	4	9·4	4
Second					R.S. virus	16	9·5	17
First	28	Bronchitis	13 months	7	R.S. virus	4	6·8	6
Second					R.S. virus	8	2·2	36
First	29	Bronchitis	6 weeks	7	R.S. virus	8	13·2	6
Second					Negative	< 4	0·9	22
First	30	Bronchitis	8 months	7	R.S. virus	< 4	2·1	10
Second					R.S. virus	8	3·6	22

* < 1/4 is considered as 1/2 for adjusting R.S. virus plaque reduction titres to 10 mg./ml. of protein.

DISCUSSION

It is now generally accepted that R.S. virus is the most important respiratory pathogen of early childhood (Chanock *et al.* 1961; Aherne *et al.* 1970). In lower respiratory tract infections it has been found that the majority of infants affected are less than 6 months of age (Elderkin, Gardner, Turk & White, 1965). At this age, maternally derived serum antibody (IgG) is present, but it does not appear to exert any protective effect. Furthermore, recent studies involving an inactivated R.S. virus vaccine showed that development of high levels of serum antibody did not prevent subsequent natural infection. Not only did vaccination fail to produce immunity, but it actually increased the severity of illness in patients who were subsequently naturally infected (Chin *et al.* 1969; Fulginiti *et al.* 1969; Kapikian *et al.* 1969; Kim *et al.* 1969*b*).

Since serum antibody does not appear to play a major protective role in R.S. virus infections, the present study has been directed at the role of local antibody present in the respiratory tract. The development of R.S. virus neutralizing activity in nasal secretions of children with R.S. virus lower respiratory tract infections has been recently described by Kim and her colleagues in 1969 and the presence of neutralizing activity in the first secretions was also demonstrated. However, these workers did not attempt to classify the R.S. virus infections into clinical categories.

The results obtained in the present study are in agreement with those obtained by Kim and her colleagues, but it was further found that a high titre of neutralizing activity was more prevalent, during the acute stage of illness, in patients with R.S. virus bronchiolitis than in patients with other types of R.S. virus infection. Although Kim and her colleagues often found neutralizing activity present in the first secretions, taken early in illness, they explained this in terms of the speed of development of nasal secretory antibody. Another possible explanation is that the neutralizing activity was already present, due to previous infection, or its rapid rise was due to previous sensitization with R.S. virus (Gardner, McQuillin & Court, 1970).

Although patients admitted to hospital with bronchiolitis often had histories of upper respiratory tract infections, these were of short duration and all were admitted within 48 hr. of the onset of acute symptoms. Speed of development of neutralizing titre in paired secretions (Table 2) which was always within 7 days, tends to support the hypothesis of a previous exposure and possible sensitization with R.S. virus antigen.

It was found that the calculation of neutralizing titre was noticeably affected by the percentage reduction of plaque count used in the calculation. If, instead of using the purely arbitrary figure of 60 % reduction, a reduction of 50 % was used in the calculation, the number of patients with a fourfold rise in neutralizing titre was increased from 4 to 5 (Table 2).

The exact nature of the neutralizing activity demonstrated is presumably antibody, and it is hoped that this impression may be confirmed by examining a larger series of patients. It is improbable that the neutralizing activity is due to

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interferon, as the rises in neutralizing titres occur at a time when antibody and not interferon would be increasing. Further studies are being undertaken to confirm that the nature of neutralizing activity is antibody. If this is so, it is unlikely to be maternal IgG, as this would not be consistent with the rising titres found in the paired secretions. Furthermore we have found, in a preliminary investigation using immunoelectrophoresis and quantitative Hyland Immuno-plates (Fahey & McKelvey, 1965), that the only type of immunoglobulin consistently present at a substantial level in the respiratory tract is IgA.

An investigation of 27 paired nasopharyngeal secretions by immunofluorescence has also been undertaken (Gardner McQuillin & McGuckin, 1970). It was found that the cells in the second secretion appeared duller on staining, as if 'blocked'; virus isolation from these secretions often proved unsuccessful. These results tend to support the conclusion that the neutralizing activity in the nasopharyngeal secretions may be due to antibody.

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