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Age related IgG subclass response to respiratory syncytial virus fusion protein in infected infants

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SUMMARY

Respiratory syncytial virus (RSV) fusion protein was purified by immunoaffinity chromatography using a mouse monoclonal antibody coupled to Affi-gel 10. The fusion protein was homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and free of other detectable viral or cellular protein. The purified fusion protein was used in a quantitative enzyme-linked immunosorbent assay (ELSIA) to measure the age-related antibody response to this protein in infected infants. The four IgG subclasses, IgA and IgM levels were determined for infants under 6 months of age, infants aged 6 months to 1 year and infants aged 1 year and over. Most infants over 6 months of age showed marked increases in both IgG1 and IgG3 antibodies with poor or negligible response with IgG2 and IgG4. By contrast infants under 6 months failed to respond by the production of IgG antibodies, although increases in IgA and IgM levels were observed. These data may explain the failure of primary RSV infections to induce protective immunity and have implications for the strategic use of attenuated RSV vaccines.

Keywords virus fusion protein antibody response antibody subclasses

INTRODUCTION

Respiratory syncytial virus (RSV) inevitably infects all infants with the primary attack resulting in a febrile pneumonitis in 50% of cases (Parrott *et al.*, 1973). In urban Britain 1 in 50 babies become sufficiently ill to require hospital treatment (Report to the MRC, 1978). Longitudinal studies of children in a day care centre (Henderson *et al.*, 1979) showed an attack rate for the first infection of 98% and for second and third infections of 75% and 65%. Of particular note was the finding that immunity resulting from a single infection had no ameliorating effect on the severity of the illness associated with re-infection 1 year later. These workers suggested that age was an important factor in the development of protective immunity.

In a detailed study of protection against RSV infection Glezen *et al.* (1981) showed that infants born with high levels of maternal IgG able to neutralize RSV developed milder illness and at a later age than infants with low antibody levels. Our group (Ogilive *et al.*, 1981) obtained comparable results using a membrane fluorescence technique. IgG antibodies against key virulence determinants on the virus might be expected to protect the lungs since levels of this immunoglobulin in the terminal airways equal the serum values (Bell *et al.*, 1981). In RSV the fusion protein (F protein, M_r 68,000) is the dominant glycoprotein in both the infected cell membrane and the virion surface. In

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order to understand the natural history of the infection and to assess the feasibility of vaccinating infants against RSV we have purified F protein and measured the age-related IgG subclass response in naturally infected babies.

MATERIALS AND METHODS

Growth and extraction of virus-infected cells. The Edinburgh strain of human RSV (Ogilvie *et al.*, 1981) was grown in HEp-2 cells maintained in Dulbecco's minimal essential medium (MEM) supplemented with 1% fetal calf serum (FCS). For the production of fusion protein, monolayers in 2·5 l roller bottles were infected with RSV at a multiplicity of infection of 10. Cells were harvested 40 h post-infection by treating each roller bottle with 20 ml 10 mM Tris-HCl pH 7·4 containing 0·18 M NaCl, 0·25 mM EDTA and 0·1 mM phenylmethylsulphonyl fluoride (PMSF) for 15 min at 4°C. Detached cells were collected by centrifugation at 5000 *g* for 15 min and resuspended at 10^8 cells/ml in lysis buffer 10 mM Tris-HCl pH 7·5, 0·1 mM EDTA, 0·1 mM-PMSF, 0·5% (v/v) Nonidet P40. After 20 min at 4°C cells were ruptured using a Dounce homogeniser, NaCl added to 0·1 M and intact cells, nuclei and virus capsid removed by centrifugation at 10,000 *g* for 30 min.

Purification of fusion protein by immuno-affinity chromatography. The procedure for the production of hybridomas secreting monoclonal antibodies reactive with RSV polypeptides has been described previously (Ward *et al.*, 1983). Briefly, BALB/c mice were immunized intraperitoneally with 10^8 RSV infected HEp-2 cells and boosted at weeks 4 and 8 and again 4 days prior to fusion. The spleen cells were fused with BALB/c NS1/1 myeloma cells; hybridomas were screened for anti-fusion protein activity and were cloned by limiting dilution. A non-neutralizing, low avidity anti-fusion protein monoclonal designated F1 was chosed for the preparation of an affinity column.

The phenotypically stable hybrid was expanded by growth in culture and approximately 10^7 cell injected intraperitoneally into BALB/c mice primed with Pristane (Sigma Chemical Co., St Louis, MO, USA). Ascitic fluid was clarified by centrifugation at 15,000 *g* for 10 min and applied to a Protein-A Sepharose column in 0·14 M phosphate buffer pH 8·2. The IgG2b monoclonal was eluted with 0·2 M sodium citrate pH 4·0 and neutralized by collection into 1 M Tris-phosphate buffer pH 7·2. Purified F1 (20 mg) was coupled to the activated affinity support Affi-Gel 10 (Bio-Rad Laboratories, Watford, UK) according to the manufacturers instruction. Unreacted *N*-hydroxy succinimide ester groups were blocked by reaction with 1 M ethanolamine pH 8·0 for 1 h at room temperature.

RSV-infected HEp-2 cells were extracted with 0·5% (v/v) Nonidet P40 (Ward *et al.*, 1983) and clarified by centrifugation at 100,000 *g* for 2 h. The NP40 extract was passed twice through the F1-monoclonal affinity column (3 ml bed volume). The column was then washed extensively with RIPA buffer (Ward *et al.*, 1983) followed by phosphate buffered saline (PBS) containing 0·5% (v/v) Brij 96 prior to elution of the bound antigen with 1 M ethanolamine (pH 10·5) containing 0·5% (v/v) Brij 96. Fractions were neutralized with ethanoic acid (acetic acid) and antigen detected by enzyme-linked immunosorbent assay (ELISA), using purified F1 monoclonal antibody. Fractions containing ELISA-positive material were pooled and the fusion protein was precipitated by the addition of three volumes of ice-cold ethanol. The precipitate was recovered by centrifugation and resuspended in water.

Patient sera. The sera used were collected from children admitted to Southampton General Hospital over the period November, 1982 to January, 1983 with signs and symptoms suggestive of RSV infection of the lower respiratory tract. Proof of infection was by direct isolation of RSV from nasopharyngeal secretion.

ELISA assay. In developing this assay system we encountered the problems of allotype restriction and cross-reactivity described by Coleman *et al.* (1985). Despite examining a wide range of commercially available mouse monoclonal antibodies it was not possible to locate equally avid antibodies to the different subclasses. To compensate for such differences we used standard curves to convert ELISA OD_{450} to antibody bound per well.

Fusion protein was diluted to a concentration of 1 μg/ml in coupling buffer (100 mM Na_2CO_3/$NaHCO_3$,pH 9·6) and 100 μl volumes added to the wells of 'high activated' PVC immunoassay plates (Flow Laboratories, Irvine, UK). After 16 h incubation antigen-coated plates were washed

four times with PBS containing 0·05% (w/v) polyoxyethylene ether W-1 (Sigma Chemical Co., St Louis, MO, USA) (PBS-W1). According to the antibody titre determined by prior screening, sera were diluted 1:50, 1:100 or 1:500 in PBS-W1 containing 5% normal rabbit serum and 0·1 mM EDTA. Six replicate tests were performed for each IgG subclass, IgA and IgM. After overnight incubation at room temperature plates were washed four times in PBS-W1 and monoclonal antibodies specific for each IgG subclass added: anti-IgG1 (Miles-Yeda, Rehovot, Israel; clone SG-16) dilution 1:500, anti-IgG2 (Miles-Yeda, Rehovot, Israel; clone SH-22) dilution 1:400, anti-IgG3 (Seward, Code BAM08) dilution 1:500 and anti-IgG4 (Miles-Yeda, Rehovot, Israel; clone SK44) dilution 1:2000. After overnight incubation at room temperature plates were washed four times in PBS-W1 and species specific Anti-mouse IgG horse-radish peroxidase linked $F(ab')_2$ (Amersham International, Amersham, UK) added at a dilution of 1:2000. After 2 h incubation at 37°C plates were washed six-fold in PBS-W1 and the colour developed using 3,3′,5,5′-Tetramethylbenzidine (Miles Scientific, Slough, UK) as the substrate. The determination of IgA and IgM was performed as a single step assay using peroxidase conjugated anti-α chains and anti-μ chains (Dakopatts, High Wycombe, Surrey, UK).

Standardization of ELISA data. World Health Organization Reference Human IgG-subclasses were obtained from Dr Skvaril (University of Berne) and human IgA and IgM from the Tenovus Research Laboratories, Southampton. These antibodies were purified by chromatography on a TSK-G2000 SW molecular sieve column in 0·1M sodium phosphate buffer pH 6·8 containing 0·1M NaCl and the central peak collected for iodination. Antibodies were radioiodinated as follows: protein was adjusted to a concentration of 1 mg/ml in PBS and mixed with 250 μCi Na ^{125}I (15 μCi/μg) in a total volume of 100 μl. A single Iodo-bead (Pearce, Rockford, IL, USA) was added and the reaction allowed to proceed for 5 min before separating unreacted iodide by chromatography on Sephadex G-25. Sequential dilutions of ^{125}I-labelled antibodies (all at 1μCi/μg) were added to the wells of PVC plates and ELISA assays carried out as described using three replicate tests for each antibody concentration. Wells were cut from the plates and bound ^{125}I-antibody determined by Gamma counting. For each IgG subclass, IgA and IgM a standard curve was constructed plotting ELISA reading (OD_{450}) against amount of antibody bound to the well.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed as described by Ward *et al.* (1983).

RESULTS

Purification of fusion protein

The preparation of fusion protein eluted from the affinity column was analysed by SDS-PAGE using a heavily loaded gel under reducing and non-reducing conditions. Under reducing conditions the gel (Fig. 1, track 2) shows a major band (VGP48) representing the heavy chain of fusion protein and two minor bands VP23 and VGP20. (These possibly represent modified light chains of the Fusion protein; Ward *et al.*, 1983); the fusion protein used appears as a single diffuse band VGP75 in unreduced gels (Fig. 1, track 3). The gels demonstrate the freedom from contamination by other viral components. Equally important no monoclonal antibody eluting from the affinity column matrix could be detected by SDS-PAGE. Eluted antibody would have reacted with the anti-mouse conjugate to give a high background in the ELISA thereby reducing the sensitivity.

Antibody response of infants to infection with RSV

Patients presented with symptoms referable to the lower respiratory tract with a mean duration of 5 days. Coryza which may or may not be due to RSV infection predated pulmonary symptoms in 75% of infants. Bronchiolitis was the respiratory syndrome seen in younger infants while some older children had additional features of broncho-pneumonia. The antibody levels in acute and convalescent sera are presented (Fig. 2 a,b) relative to the time of onset of lower respiratory tract symptoms with the data expressed graphically to permit comparison of age-related IgG subclass, IgA and IgM response to fusion protein. Most infants under 6 months responded to RSV infection with the production of IgA and IgM antibodies to the virus F protein but comparable increases in IgG sub-classes were not detected. By contrast infants over 1 year and most within the 6 to 12

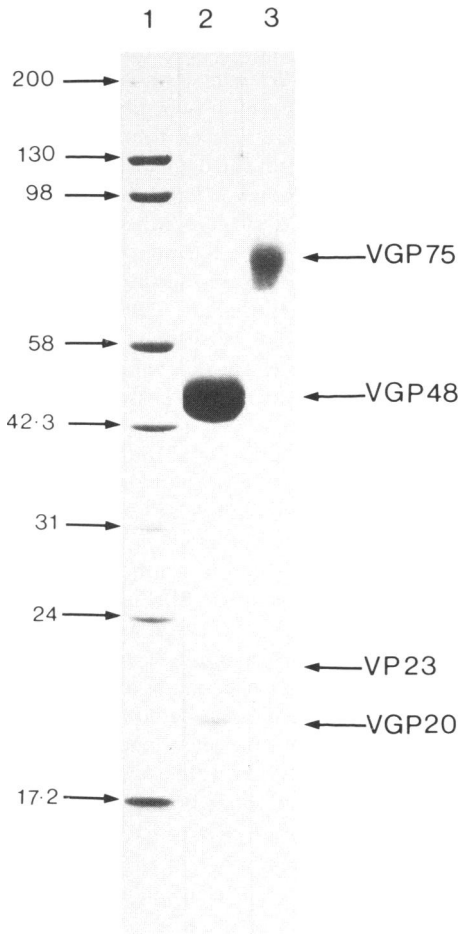

Fig. 1 SDS polyacrylamide gradient gel of immunoaffinity purified RSV fusion protein. Track 1, molecular weight standards; track 2, fusion protein derivatised for electrophoresis by reduction with mercaptoethanol; track 3, fusion protein prepared for electrophoresis in the absence of reducing agent.

months age group showed marked increases in both IgG1 and IgG3 antibodies. Despite the significant increase in IgG1 titres to F protein in convalescent infants over 1 year the absolute values did not exceed the maternal levels measured in infected babies under six months. For older infants with raised IgM levels in the acute phase sera, upper respiratory tract infection pre-dated hospital admission.

DISCUSSION

We have chosen to study the antibody response to RSV fusion protein because monoclonal antibodies to this protein neutralize the virus and inhibit syncytial formation (Walsh & Hruska, 1983). In passive immunization studies such monoclonal antibodies protect the lungs of mice challenged intranasally with virulent RSV (Taylor *et al.*, 1984). If, during natural infection human antibodies to F protein protect by neutralizing the virus, blocking cell to cell spread or the release of infectious viruses, individual immunity should be a function of the concentration of antibody in the lower respiratory tract, antibody avidity and epitope specificity. Activation of effector mechanisms such as complement mediated lysis of infected cells or antibody dependent cell cytotoxicity will

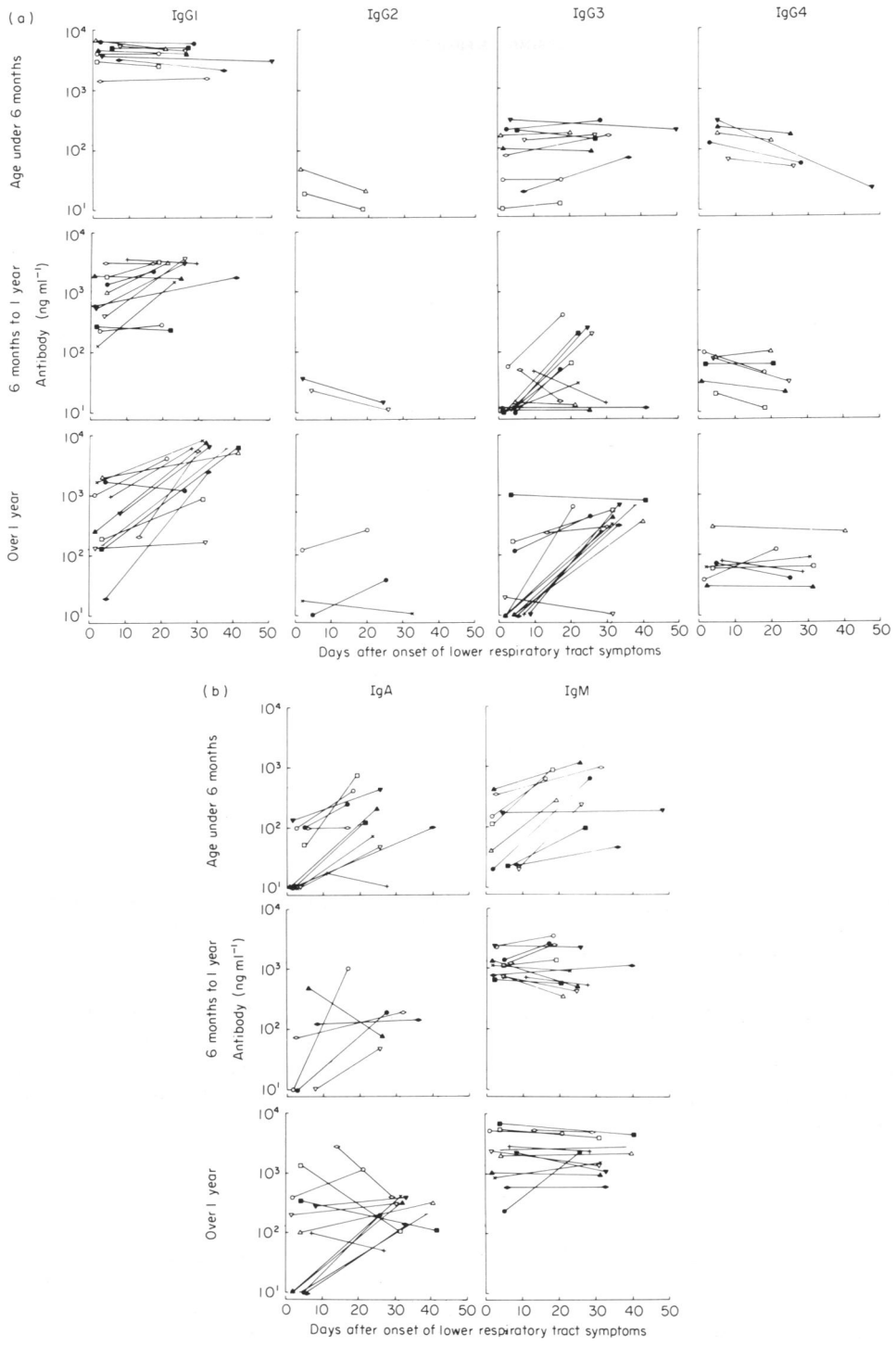

Fig. 2 Age related antibody response to RSV fusion protein. Individual patients in each age group are represented by the same symbol. Undetectable antibody responses for a given patient are ommitted from the graphs. For ease of visualization the acute and convalescent titres for each patient are connected as straight lines on the graphs. (a) IgG subclass responses. (b) IgA and IgM responses.

depend largely upon the presence of IgG1 or IgG3 antibodies. Analysis of broncho-alveolar lavage fluid from adults has shown that the concentrations of IgG1 and IgG2 in the terminal airways equals the serum levels (Merrill *et al.*, 1985).

Our data show that antibodies to F protein in the acute phase serum of infected babies under 6 months of age are predominantly IgG1. Obviously then the presence of antibodies reactive with a key virulence determinant of RSV and of an IgG subclass able to activate effector mechanisms do not confer complete protection to the lung. This may be a concentration related phenomenon since serious RSV infection is rare in the first 6 weeks of life and infection is milder in infants receiving the highest dose of neutralizing antibody from the mother. In babies under 6 months of age with bronchiolitis we were unable to detect the production of IgG1 antibodies to F protein and the convalescent titre in older infants was comparable to that in infected babies. Several factors may be involved in this limited response. First the antigenic load may be sub-optimal perhaps because viral replication is partially suppressed by maternal antibodies. Certainly attenuated RSV vaccine given parenterally produced no antibody response in children while maternal antibody persists and in experimental animals the response can be suppressed by passively administered antibodies (Belshe *et al.*, 1982; Prince *et al.*, 1982). Immunological immaturity of the younger infants studied could be a further determinant of the magnitude of the antibody response to RSV fusion protein. The IgG1 and IgG3 antibodies detected in the infants studied were the subclasses which mature earliest in childhood (Oxelius, 1979).

Our results for IgG subclasses reactive with RSV fusion protein are compatible with the findings for other enveloped viruses such as rubella (Skvaril & Schilt, 1984), Herpes simplex and zoster (Sundqvist, Linde & Wahren, 1984; Coleman *et al.*, 1985), although the target antigens of the antibodies concerned were not determined. IgG3 antibodies to these viruses were found in acute phase sera decaying away over the next 6 months. The IgG3 antibodies to RSV F protein found in the serum of almost all infected infants may be important in control of the disease process. The finding that IgG3 concentrations in the lungs of smokers exceeds the serum value implies local antibody synthesis (Merrill *et al.*, 1985) and the fact that IgG receptors on alveolar macrophages show the greatest affinity for IgG3 (Naegel, Young & Reynolds, 1984) implies an important role for this subclass in lung defence. Merrill also found IgG4 antibody levels in the lung exceed the serum level and IgG4 antibodies reactive with viruses have been described (Linde, 1985). One preliminary report (Bui *et al.*, 1982) suggests that raised levels of IgG4 together with IgE were present in the serum of infants during the acute phase of bronchiolitis and by interaction with mast cells contributed to the bronchospasm. We were unable to detect IgG4 antibodies to F protein in most infants with bronchiolitis but this does not exclude the possibility of IgG4 antibodies reactive to other virus components.

The prevention of RSV infection in the first months of life is a priority area in vaccine development. Earlier attempts to immunise infants against RSV infection with formalin-killed vaccine not only failed to protect but induced enhanced severity and frequency of disease on subsequent exposure to natural infection (Fulginitti *et al.*, 1969; Kim *et al.*, 1969). The immunological basis of disease enhancement is unclear but RSV immune complexes react with polymorphs to release thromboxanes and superoxide (Faden, Kaul & Ogra, 1983). Purified F protein vaccines are being developed in several laboratories in the expectation that such a vaccine would circumvent the disease enhancement problem. However, such vaccines cannot be tested without more detailed studies of the antibodies reactive with different epitopes on F protein and the role in the prevention or enhancement of infection.

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