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Class-specific antibodies to bovine respiratory syncytial virus in experimentally infected lambs

R. SHARMA* AND Z. WOLDEHIWET†

University of Liverpool, Department of Veterinary Pathology, Veterinary Field Station, Leahurst, Neston, Wirral L64 7TE, UK

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SUMMARY

Enzyme-linked immunoabsorbent assay (ELISA) was used to titrate virus-specific IgG, IgM and IgA levels in nasal secretions, lung lavage fluids and serum samples sequentially obtained from lambs experimentally infected with bovine respiratory syncytial virus (RSV). Virus-specific IgG and IgM responses were measured by the indirect double antibody sandwich ELISA using anti-bovine RSV monoclonal antibody, as capture antibody, and peroxidase-conjugated anti-sheep IgG and anti-sheep IgM. Virus-specific IgA antibodies were measured by antibody capture assay using anti-sheep IgA (α-chain specific) and anti-bovine RSV monoclonal antibodies.

Bovine RSV-specific IgM and IgA antibodies were detected in the serum samples within 6 days post-inoculation (p.i.). Virus-specific IgC antibodies appeared in serum samples 4 days later. In nasal secretions, IgA antibodies appeared 7 days p.i. but IgM antibodies were not detected until 12–16 days p.i. In serum samples, IgM titres were predominant for the first 2 weeks p.i. IgC titres becoming predominant thereafter. In nasal secretions and lung lavage fluids, IgA titres were significantly higher than IgM or IgG titres up to 21 days p.i. (0·01).

INTRODUCTION

Bovine respiratory syncytial virus (bovine RSV) is one of the most important respiratory tract pathogens of calves [1]. Despite the manifestation of severe disease in natural infections, it has been difficult to reproduce the disease experimentally in calves [2, 3]. The role of bovine RSV as cause of natural respiratory disease in sheep is not known but the virus has been isolated from naturally infected sheep [4] and neutralizing antibodies to bovine RSV are widespread in sheep and goats [5–8]. Because of their physiological similarity to calves, their susceptibility to experimental infection with bovine RSV, their low cost and short generation time, lambs are increasingly being used as an experimental model to study the disease [9–12]. Although experimental infection with bovine RSV causes only mild clinical disease, lambs experimentally infected with bovine RSV are more susceptible to secondary infection with *Pasteurella*

* Present address: Department of Veterinary Microbiology, College of Veterinary Science, Haryana Agricultural University, Misur-125004, India.
† Author for correspondence.

haemolytica [9, 13] probably because infection with bovine RSV alters the lymphocyte subpopulations and depresses lymphocyte responses to other antigens [14, 14]. We have recently shown that lambs experimentally infected with bovine RSV produce virus-specific cytotoxic T cells and neutralizing antibodies against bovine RSV [12, 16]. In this study we examined the class-specific antibody responses of lambs experimentally infected with bovine RSV.

MATERIALS AND METHODS

Bovine respiratory syncytial virus

A strain of bovine RSV (BRSV 66), passaged in lamb testis cells (LTC) three times, was maintained and used as previously described [12].

Lambs

Thirty-four 6- to 8-week-old conventionally reared Suffolk crossbred lambs, free of neutralizing antibodies to bovine RSV, were used. Twenty-two lambs were infected with bovine RSV and 12 controls injected with non-infected tissue culture fluid as previously described [12].

Collection of serum and nasal samples

Serum samples were collected from 8 lambs infected with bovine RSV and from 8 control lambs on 0, 6, 10, 14, 21, 35 and 60 days post-inoculation (p.i.).

Nasal samples were collected from 8 bovine RSV-infected and 8 control lambs before infection and at 7, 12, 16 and 21 days p.i. by placing sterile gauze packs into the nasal cavities of lambs with the help of forceps. After 10 min the swabs were taken out, the contents of the gauze squeezed into sterilized vials using a 5 ml plastic syringe and stored at −70 °C until required for analysis. Nasal secretions were screened for blood contamination by the urine test (BM test-7, Boehringer Mannheim GmbH Diagnostica, Germany).

Lung lavage

Fourteen experimentally infected lambs were killed with an overdose of pentobarbitone sodium administered intravenously; 2 lambs each on day 3 and 11, and 5 lambs each on day 5 and 8 p.i. Four control lambs were also killed. The lungs were lavaged with 250 ml of cold Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution containing 0·2% EDTA but without phenol red as described by Davis and Penwarden [17]. Lavage fluid was centrifuged at 700 ***g*** for 10 min at 4 °C and the supernatant collected for estimation of virus-specific antibodies and albumin. The albumin in lung lavage fluids was estimated by the bromocresol-green method using commercial kits (Boehringer Mannheim GmbH Diagnostica, Germany) according to manufacturer's instructions.

Bovine RSV antigen

Secondary LTC cultures were infected with bovine RSV as described previously [12]. Infected cultures were harvested after 5 days of incubation at 37 °C and the virus released by freezing and thawing. The cellular lysate was clarified by centrifugation at 1000 ***g*** for 20 min and the supernatant used as antigen for ELISA. Uninfected cells were used to prepare control antigen.

Virus neutralization test

Serum samples collected before inoculation and on 6, 10, 14, 21, 35 and 60 days p.i. were heat-inactivated at 56 °C for 30 min and examined for neutralizing antibodies to bovine RSV using calf testis cell cultures (CTC) in a mictrotitre virus-neutralization (VN) test [18]. VN titres were recorded as the reciprocal of the highest serum dilution that inhibited cytopathic effects of 100 $TCID_{50}$ of bovine RSV.

Enzyme-linked immunosorbent assay (ELISA)

Bovine RSV-specific IgG and IgM antibodies in nasal secretions, serum samples and lung lavage fluids were titrated in an indirect double antibody sandwich assay (IDAS), using a mixture of monoclonal antibodies (Mab) specific to the F- and N-proteins of bovine RSV as coating antibody, and peroxidase-conjugated IgC fraction of swine anti-sheep IgG or swine anti-sheep IgM (Evai Bios Lab., West Sussex, England). The anti-bovine RSV Mabs were kindly provided by Dr F. Westenbrink, Central Veterinary Institute, Lelystad, The Netherlands. Virus-specific IgA antibodies were detected by the antibody capture assay (ACA) method, using IgG fraction of swine anti-sheep IgA (α-chain specific) (Eivai Bios Lab., West Sussex, England), as capture antibody, and anti-bovine RSV Mab. The ELISA test was performed as described by Kimman and colleagues [19] with some modifications, using 0·05 M carbonate-bicarbonate (pH 9·6) as coating buffer, 0·5 M Tris buffer (pH 7·4) containing 1 mM-EDTA, 1 M-NaCl, 0·1% BSA and 0·05% Tween 20 as sample diluent and 0·1 M-PBS-Tween 20 (0·05%) as conjugate diluent. The substrate was 0·04% *O*-phenylenediamine (OPD) and 0·015% H_2O_2 in citrate-phosphate buffer (pH 5). After each step plates were rinsed ten times with demineralized water containing 0·25% Tween 20, 0·025% Tween 80 and 0·87% NaCl (ELISA wash).

Indirect double antibody sandwich assay

Micro ELISA polyvinyl plate (PVC, EIA plates, Flow Lab) were coated with 50 μl of the anti-bovine RSV Mab preparation, at an optimal dilution. After overnight incubation at 4 °C Mab was poured off and the remaining free binding sites on the wells were blocked by filling each well with 100 μl of 0·2% bovine serum albumin (BSA Fraction V, Sigma chemicals) in sample diluent. After 1 h incubation at 37 °C the BSA solution was poured off and the plates rinsed in ELISA wash. Bovine RSV antigen preparations (50 μl/well) and appropriate controls were then dispensed into antibody-coated wells and the plates incubated for a further 1 h at 37 °C. Fourfold dilutions of the serum samples, nasal secretions and lung lavage fluids and negative and positive controls were added in duplicates and the plates incubated for 1 h at 37 °C.

Fifty μl of optimally diluted peroxidase-conjugated antibodies against sheep IgG or IgM were added to each well, except substrate controls, and the plates incubated for 1 h at 37 °C. Finally, 100 μl of freshly prepared substrate solution were added to each well and the plates left in the dark at room temperature until the development of colour in positive serum samples. The reaction was stopped by the addition of 50 μl of 4N-H_2SO_4 per well. The absorbance of each well was

determined by a micro plate reader (MR 700, Dynatech) using a test wave length of 490 nm, calibration setting of 1·00 and a threshold of 1·99. Blank readings were obtained with substrate controls.

The antibody titre was taken as the highest dilution scoring 1 matrix unit above the value obtained with the standard negative serum in bovine RSV antigen-coated wells in the same plate as described by Kimman and colleagues [19]. The matrix for each plate was set according to the maximum E 490 value obtained with the standard positive serum. One matrix unit corresponds to $\frac{1}{10}$ of the maximum value obtained with standard positive serum sample. The titre of the test sample was taken as the highest dilution showing one matrix unit above the value obtained in the lowest dilution of standard negative sample (1/4).

Antibody capture assay

For the ACA, ELISA plates coated with optimally diluted α-chain-specific, swine anti-sheep IgA. The plates were then incubated with serial four-fold dilutions of test samples or controls, followed by bovine RSV antigen, anti-bovine RSV Mab, biotinylated anti-mouse IgC, ABC complex (ABC kit, Sera Lab, Sussex, England) and substrate solution, in that order, as described earlier in the IDAS assay. Biotinylated antibody and ABC complex were used according to manufacturer's instructions.

Analysis of data

Sequential changes in the patterns of class-specific antibody responses were analysed by analysis of variance. Titres of class-specific immunoglobulin were compared by Student's t test.

RESULTS

Lambs experimentally infected with bovine RSV showed mild clinical signs characterized by slight serous nasal discharge, coughing and pyrexia as previously reported [12]. Bovine RSV was isolated from nasal swabs obtained from 4 of 8 lambs infected with bovine RSV and from lung and tracheal tissues of the 14 bovine RSV-infected lambs killed 3–11 days p.i. [12].

Virus neutralizing antibodies

Virus neutralizing antibodies were first detected in serum samples 6 days after infection and continued to be detected until the last day of sampling (Fig. 1). Peak titres were attained 14–21 days p.i. No VN antibodies were detected in control lambs.

Purity of nasal secretions and lung lavages

Nasal secretions were negative for haemoglobin when tested by urine strip test. Lung lavage samples were negative for albumin and haemoglobin.

Standardization of class-specific ELISA

Checker-board titrations were used to establish optimum concentrations of coating antibodies, bovine RSV antigen and conjugates. All subsequent tests were performed using bovine RSV Mab diluted 1/2000, α-chain-specific swine anti-

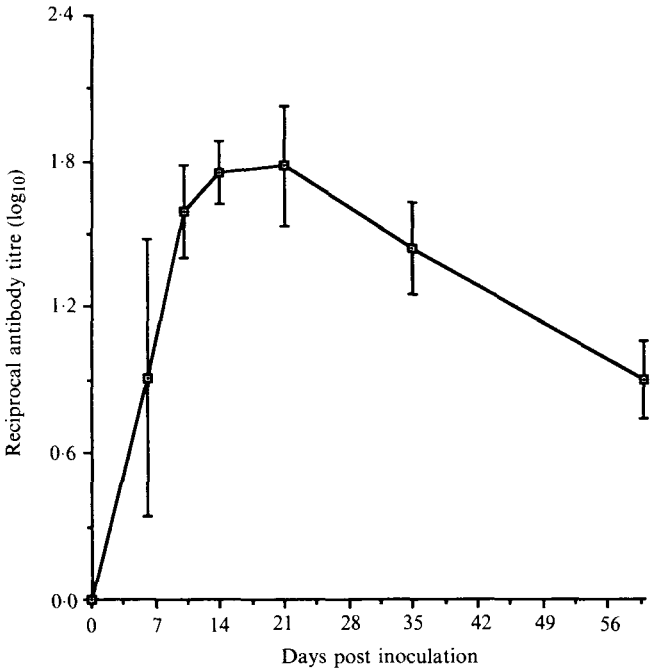

Fig. 1. Mean (±S.D.) virus neutralizing antibody titres (log_{10}) of serum samples obtained from eight lambs infected with bovine RSV.

sheep IgA diluted 1/250 for optimum coating and peroxidase-conjugated swine anti-sheep IgG and IgM diluted 1/2000 and 1/1500 respectively. Non-specific background signals were not observed in any of the controls. To test the reproducibility of the ELISA, 8 positive serum samples and 8 negative controls were tested 3 times. There was no significant variation in the titres recorded on different weeks.

Primary antibody responses

There were no detectable class-specific antibodies to bovine RSV ($<10^{0·6}$) in serum samples obtained before infection. After infection, virus-specific IgM and IgA antibodies were detected as early as 6 days p.i. but IgG antibodies appeared 6–10 days p.i. IgA antibodies were detected up to 21 days p.i. but IgM and IgG antibodies continued to be detected up to 35 and 60 days p.i. respectively (Fig. 2). Peak virus-specific IgM responses occurred 10 days p.i. but IgG titres became predominant 21 days p.i. (Fig. 2).

Local antibody responses

Nasal secretion

There were no detectable class-specific antibodies to bovine RSV ($<10^{0·6}$) in nasal secretions obtained before infection. Virus-specific IgA antibodies were first detected 7 days p.i. in nasal secretions of all eight bovine RSV-infected lambs. Peak IgA titres occurred 12 days p.i., followed by significant reductions 16 days p.i. and a significant rise 21 days p.i. (Fig. 3) $P < 0·01$). Virus-specific IgM

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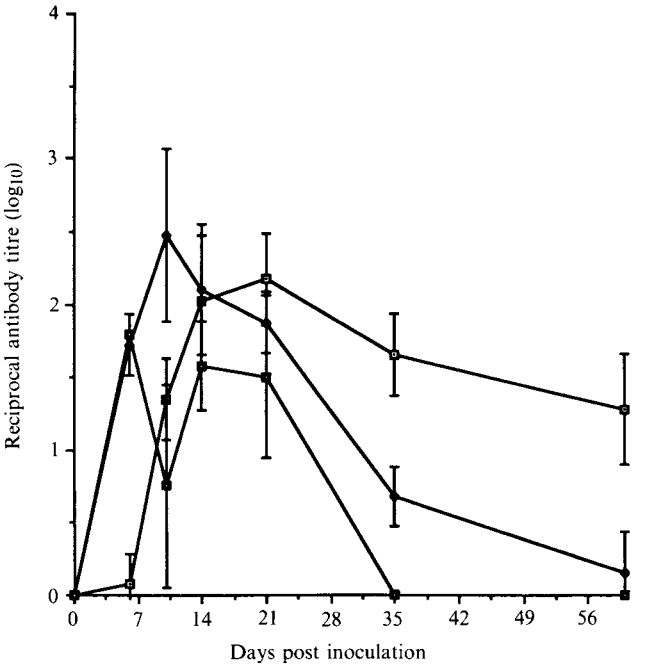

Fig. 2. Mean (±S.D.) bovine RSV-specific IgM (◆, IgG (⊡) and IgA (■) titres (log_{10}) of serum samples obtained from eight infected lambs.

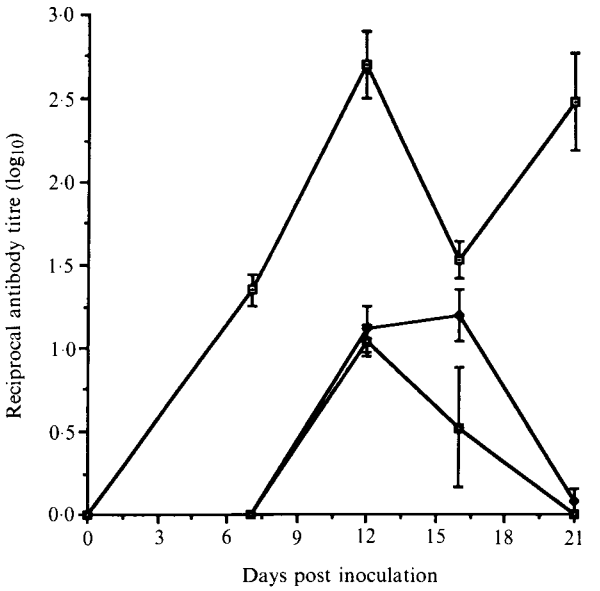

Fig. 3. Mean (±S.D.) bovine RSV-specific IgM (◆), IgG (■) and IgA (⊡) titres (log_{10}) in nasal secretions of eight infected lambs.

antibodies were detected in all bovine RSV-infected lambs 12 and 16 days p.i. but only one lamb had detectable IgM antibodies 21 days p.i. Twelve days p.i., all the bovine RSV-infected lambs had low levels of virus-specific IgG in their nasal secretions but only 6 of 8 lambs had detectable IgG antibodies 16 days p.i. No

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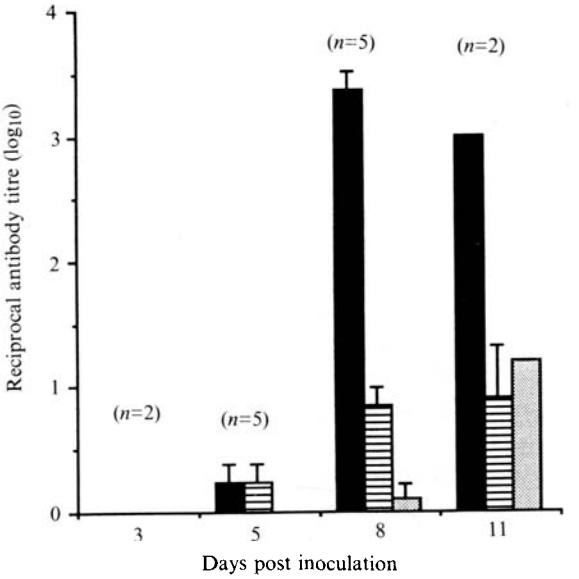

Fig. 4. Mean (±S.D.) Virus-specific IgM (▤), IgG (▨) and IgA (■) titres (log_{10}) in lung lavage fluids obtained from bovine RSV-infected lambs.

virus-specific IgG was detected in any of the lambs 21 days p.i. IgA titres were significantly higher than both IgG and IgM titres throughout ($P < 0{\cdot}05$). There were no significant differences between IgG and IgM titres 12 days p.i. ($P > 0{\cdot}05$) but 16 days p.i., IgM titres were significantly higher than IgG titres ($P < 0{\cdot}05$).

Lung lavage

No virus-specific IgG or IgM were detected in lung lavage fluids obtained from control lambs or bovine RSV-infected lambs killed 3 days p.i. Low titres of virus-specific IgA and IgM were recorded in 2 of 5 lambs killed 5 days p.i. Virus-specific IgA were detected in lung lavage obtained from 5 of 5 bovine RSV-infected lambs killed 8 days p.i. and from 2 of 2 lambs killed 11 days p.i. (Fig. 4). IgA titres were significantly higher than IgM titres ($P < 0{\cdot}01$). One of 5 lung lavage fluids obtained from lambs killed 8 days p.i. and 2 of 2 lambs killed 11 days p.i. had detectable anti-bovine RSV-specific IgG antibodies (Fig. 4).

Virus-specific IgM, IgG and IgA antibodies were not detected in the serum samples, nasal secretions or lung lavage fluids obtained from control lambs.

DISCUSSION

With the indirect double antibody sandwich ELISA, we were able to differentiate between virus-specific IgM and IgG antibodies. No cross-reactivity between virus-specific IgM and IgG was observed. This was supported by the fact that IgM antibodies but no IgG antibodies to bovine RSV were detected in serum samples obtained 6 days p.i. and IgG antibodies but no IgM antibodies to bovine RSV were detected in serum samples obtained 60 days p.i.

An analysis of the virus-specific IgM levels in serum samples sequentially collected from lambs experimentally infected with bovine RSV revealed that

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virus-specific IgM antibodies appear as early as 6 days after infection and persist up to 35 days p.i. Peak IgM titres were recorded 10 days after infection. In human infants, Welliver and colleagues [20] detected IgM antibodies to RSV in 5 of 22 children within 4 days of illness and Cevenini and colleagues [21] found IgM antibodies to RSV in 3 of 36 serum samples within 4 days of illness but IgM antibodies to RSV were detected in most of the serum samples obtained 10–14 days after the onset of illness. In calves, Kimman and colleagues [22] detected bovine RSV-specific IgM in 6 colostrum-deprived calves 8–10 DPI but peak IgM tires were recorded 10–12 days p.i. Thomas and colleagues [23] detected bovine RSV-specific IgM antibodies in 6 of 7 serum samples collected from gnotobiotic calves at the time of slaughter, 9–14 days after experimental infection. The decay of IgM antibodies to bovine RSV observed in the present study was similar to that reported by Kimman and colleagues [22] in colostrum-deprived calves infected with bovine RSV.

The diagnosis of bovine RSV infection by virus isolation is difficult [23–25]. Usually the diagnosis of bovine RSV infection is based on seroconversion [25, 26]. Therefore, the detection of virus-specific IgM has been advocated for the presumptive diagnosis of RSV infection [20, 22].

There are conflicting reports on the presence of RSV-specific IgA in serum samples of naturally infected infants and experimentally infected calves. Welliver and colleagues [20] found virus-specific IgA in 5 of 41 serum samples obtained from human patients in the first 9 days of illness but reported a rise in virus-specific IgA titres in the whole group of patients 10–14 days after the onset of illness. In contrast, Cranage and Gardner [27] could not detect virus-specific IgA antibodies in all but one serum sample obtained from 14 infants during the acute phase or convalescent phase of natural infection with RSV. Kimman and colleagues [22] detected virus-specific IgA in 6 of 6 calves 8–10 days after experimental bovine RSV infection. However, Thomas and colleagues [23] did not detect bovine RSV-specific IgA in any of the serum samples obtained from 8 calves at the time of slaughter, 7–14 days after experimental infection. In the present study, virus-specific IgA was first detected 6 days after infection and continued to be detected up to 21 days p.i.

In respiratory viral infections, resistance is more closely related to high titres of nasal antibody than to serum antibody [28, 29]. RSV-neutralizing antibodies have been demonstrated in nasal secretions obtained from naturally or experimentally infected human subjects and experimentally infected calves [30–32]. Class-specific antibody responses to RSV in nasal secretions of naturally infected human infants and experimentally infected calves have been detected by the indirect fluorescent antibody test and ELISA [22, 33, 34]. In the present study, bovine RSV-specific antibody titres were determined by ELISA using the end-point titration method. The end point titration method has generally been preferred to the single dilution method because it is probably less influenced by antibody affinity and antibody titre [35–37].

An analysis of virus specific immunoglobulins in nasal secretions of bovine RSV-infected lambs revealed that virus-specific IgA was the predominant antibody. Virus-specific IgA was first detected 7 days after infection. Peak IgA titres occurred 12 days p.i., followed by a significant reduction 16 days p.i. and a

secondary rise 21 days p.i. In human infants, Hornsleth and colleagues [29] detected RSV-specific IgA antibodies in nasal secretions obtained from 19 of 22 infants 5–8 days after the onset of illness. McIntosh and colleagues [33] first detected cell-free IgA antibodies to RSV in 21 of 22 nasal secretions 3–4 days after hospitalization but peak secretary IgA titres were detected 9–10 days after hospitalization. In bovine RSV-infected calves, the kinetics of class-specific immunoglobulins in respiratory secretions is not well established. We are aware of only one report in which virus-specific IgA antibodies were detected 10–13 days after experimental infection of calves with bovine RSV [22].

Several workers detected virus-specific IgG in nasal secretions of human infants after natural infection with RSV [33, 38] but Kimman and colleagues [22] attributed virus-specific IgG in nasal secretions of experimentally infected calves to blood contamination. In the present study virus-specific IgG was detected in nasal secretions of experimentally infected lambs 12 and 16 days p.i. The IgG detected in secretions was not due to blood contamination because all the secretions used for analysis were found to be negative for haemoglobin. Several workers have shown that both IgA- and IgG-secreting cells are present in the nasal mucosa. Morgan and colleagues [39] showed that there were both IgG1-secreting and IgA-secreting cells in nasal mucosa. Alley and colleagues [40] found IgA-, IgG- and IgM-bearing cells in nasal mucosa, bronchial mucosa and lungs of 2-month-old lambs. IgA-bearing cells were predominant throughout the respiratory tract.

It appears that RSV-specific IgG and IgM antibodies are short-lived but IgA antibodies persist for prolonged periods in nasal secretions. This is probably due to the emergence of predominantly IgA-secreting B cells in mucosal surfaces determined by specific T helper cells which induce IgM-bearing B cells to switch into IgA bearing cells and suppressor cells directed at IgG or IgM immunocytes [41].

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