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UPPER RESPIRATORY TRACT EPITHELIAL CELLS AS A COMPONENT OF LOCAL IMMUNITY AGAINST INFLUENZA

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A conjugate of influenza virus and erythrocytes was used to detect and quantitatively determine the occurrence of respiratory epithelial cells carrying surface-associated antibody. The method was specific since uncoated erythrocytes, or erythrocytes coated with heterologous virus failed to attach.

Epithelial cells carrying surface-bound anti-influenza virus antibodies (Ab) were found in the upper respiratory tract of 23 influenza patients and 117 healthy persons during and after an influenza A(H1N1) outbreak, and in 13 volunteers immunized with killed influenza A(H1N1) vaccine. Furthermore, the appearance of Ab-carrying cells correlated with that of circulating and secretory Ab in washings from the same individuals. The results suggest that the respiratory epithelial cells play an important role in the development and function of local anti-influenza immunity.

respiratory epithelium influenza secretory antibody local immunity

INTRODUCTION

The concept that mucosal immunity plays an important role in acquired resistance against infection has recently received extensive experimental support [1,2,4]. Although there is some consensus that local immunity is primarily mediated by secretory Ab rather than by cell-mediated immunity, very little, if any, interest has gone to the possibility that there may exist a close relationship between these immunoglobulin molecules and epithelial cells. Determinations of secretory Ab were mostly done on mucosal washings [4]. The collection of such washings and their further treatment cannot adequately be standardized. Moreover, the presence of Ab in washings, whatever their concentration may be, does not give information as to whether they are solubilized in the mucus or linked to the cell surfaces.

Using antisera and complement it was first shown in 1975 [5] that sIgA was attached to the surface of respiratory epithelial cells, and that influenza virus, added in vitro, could

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chase this Ab. It was further demonstrated in influenza virus-infected rats [7] that the cell-associated Ab were antigen-specific and their presence was related with cellular resistance against virus infection. Specific secretory Ab bound to the surface of epithelial cells have also been demonstrated in children with respiratory syncytial virus infection [3].

In the course of an epidemiological and immunological survey of an influenza outbreak we have used a modification of the indirect haemagglutination (HA) test for the detection of anti-influenza virus Ab bound to respitory epithelial cells. This study allowed us to describe the kinetics of appearance of Ab-carrying cells during and after an influenza A(H1N1) infection in man.

MATERIALS AND METHODS

Subjects

Our study was done on 23 persons with clinically manifest influenza, confirmed by virus culture, during an A(H1N1) outbreak (patients). The study also comprised 117 healthy persons in the post-epidemic period (convalescents), and 13 healthy volunteers immunized intranasally with killed influenza A(H1N1) vaccine (volunteers). All the individuals of the second group were born after 1956, the year when influenza A(H1N1) went out of circulation. Although it was not known whether they had suffered from clinical influenza A(H1N1) we considered them convalescents because of their high initial serum antibody titers (1/128 and higher), an indication that they had been in contact with the virus. Cells, nasal washings and sera were collected from all individuals in each group at different time intervals, as described below.

Conjugation of influenza virus with sheep red blood cells (SRBC)

A virus-erythrocyte conjugate (v-SRBC) was obtained using 3,3′-diaminobenzidine (DAB) tetrahydrochloride as a linker. SRBC were first formalinized according to Weinbach [8]. Then 0.1 ml aliquots of a 50% suspension of formalinized SRBC were added to equal volumes (1 ml) of virus suspension (A/USSR/90/77(H1N1) strain or A/Hong-Kong/I/68(H3N2) strain concentrated to a haemagglutination titer of 1/2048) together with increasing volumes (0.1–1.5 ml) of DAB solution (1 mg/ml). After 30 min incubation at 37°C the different conjugates were washed three times with phosphate-buffer saline (PBS). The optimal amount of DAB that served for the preparation of the conjugate used in all furhter experiments was determined using a test anti-influenza antiserum. Each conjugate was tested using serial dilutions of the serum in an U-shaped microtiter tray. The last well of each row contained only PBS to indicate non-specific agglutination. The conjugate that gave an agglutination end-point at dilution 1/64–1/128 was chosen for testing Ab-carrying epithelial cells. For testing Ab in serum and washings the conjugate giving an agglutination end-point of 1/1024–1/2048 was preferred.

Assay of cell-bound Ab

Respiratory epithelial cells were obtained by rubbing the inferior nasal meatus with a dry cotton swab. The swab was immediately immersed in medium 199. After centrifugation at 500 r.p.m. for 3 min, the sedimented cells were resuspended in 0.05 ml of medium 199 to which an equal volume of v-SRBC was added. After 20 min incubation at 18°C, the cells were introduced in a Bürker chamber and counted under phase-contrast microscope. Cells that had fixed at least three v-SRBC were considered positive. The number of cells counted varied depending on the total amount of collected cells; therefore, the number of positive cells was expressed as percentage of all counted cells per sample.

In cells of influenza patients, sera and washings were collected at the onset of the disease as well as in the early and late convalescent periods. In the group of convalescents, samples were taken every third month after the peak of the outbreak. Volunteers were examined only twice: before and 12–14 days after the immunization.

As a control, cells taken at each time interval were also incubated with non-conjugated formalinized SRBC, or with A(H3N2) virus conjugates.

Sera. Blood was collected from the cubital vein. The sera were stored at -20°C until examination. Ab concentrations were determined by the indirect HA test.

Washings were collected and assayed for Ab content by the method described by Shvartsman et al. [6].

RESULTS

v-SRBC were found attached around the apical extremity of the Ab-positive cells, i.e. on the superolateral cell surface, in the proximity of the junctional complexes. It cannot be certified whether this in vitro position reflects the in vivo situation, in that the cells may have been modified by the centrifugation.

The quantitative results of the survey are represented in Table 1. Since all countings were done under standard conditions, the number of cells counted per individual at the different time points reflected the readiness of the cells to be removed from the epithelium. Thus, during the period of active disease, the number of cells in the samples were significantly higher than in samples collected later on. Similarly, low numbers of cells were also harvested from convalescents and from immunized volunteers in whose respiratory tract no virus replication occurred.

The results of Table 1 show that 2–3 days after the onset of the disease in patients only small numbers of cells exhibited v-SRBC on their surfaces. On days 12–14, however, the proportion of Ab-carrying cells had increased about 8-fold ($P < 0.01$); on day 90 the percentage of positive cells was already 16-fold higher than initially. The Ab concentration in washings increased similarly between the first and second time points tested, but on the third time point high titers remained only in 36.4% of the examined individuals. Thus, Ab-carrying epithelial cells and secretory Ab in washings showed a parallel

TABLE 1

Antibody-carrying epithelial cells in the respiratory tract as compared to secretory and circulating antibody in influenza patients, convalescents and vaccinated volunteers

Group of individuals	No. of persons in the group	Time of sampling (after infection or vaccination)	Antibody-carrying cells				Antibody in nasal washings [$-\log_2$ GMT (±S.E.M.)]	Circulating antibody [$-\log_2$ GMT (±S.E.M.)]
			Average no. of cells available for examination (per individual)	Average no. of positive cells[a]	% Positive	No. of v-SRBC per positive cell (±S.E.M.)		
Patients	23	2– 3 days	385	2.60	0.67	3.3 (0.08)	2.2 (0.5)	5.3 (0.3)
		12–14 days	178	9.74	5.47	6.8 (0.31)	3.5 (0.1)	7.3 (0.4)
		90–95 days	89	9.45	10.60	6.2 (0.41)	2.6 (0.2)	6.8 (0.3)
Convalescents	117	3 months	80	5.45	6.81	5.0 (0.31)	2.7 (0.1)	6.7 (0.4)
		6 months	92	1.16	1.26	3.5 (0.25)	1.4 (0.1)	6.3 (0.4)
		9 months	88	1.40	1.59	3.4 (0.21)	1.2 (0.1)	6.1 (0.4)
Vaccinated volunteers	13	0 day	88	1,80	2.04	4.8 (0.50)	1.4 (0.2)	3.4 (0.3)
		12–14 days	93	11.20	12.04	5.2 (0.37)	5.4 (0.2)	6.8 (0.2)

[a] Epithelial cells with minimally three v-SRBC attached.

GMT, geometric mean titer; S.E.M., standard error of mean.

increase only in the early convalescent period; in the late convalescent period the proportion of Ab-coated cells was still increasing, while the concentration of the Ab in the washings was decreased. As shown in Table 2 the kinetics of serum Ab levels in this group of individuals was moderately well correlated (r = 0.46) with the kinetics of prevalence of Ab-carrying epithelial cells. Circulating Ab levels, on the other hand, correlated very well (r = 0.89) with those of secretory Ab at the first two time points. This correlation reveals a normally responding humoral immune system and suggests that both local and systemic immune responses are involved in anti-influenza immunity [2,4,6].

In the group of convalescents, a relatively high percentage of Ab-carrying cells was noted 3 months after the peak of the outbreak (Table 1). However, the score was smaller than that seen in the group of patients 3 months after disease. Moreover, samples collected 6 and 9 months after the peak of the outbreak contained very low numbers of Ab-carrying cells, as well as low titers of free Ab in the washings. The number of v-SRBC per cell was highest in the early convalescent period and remained high up to the third month. At the end of the period under investigation the number of v-SRBC bound per cell was restored to its initial value. Thus, the kinetics of this parameter were similar to the kinetics of the prevalence of Ab-carrying cells.

Two weeks after immunization of healthy volunteers the percentage of Ab-carrying cells increased 6-fold. The change in number of v-SRBC bound per cell was insignificant, perhaps because of the relatively high initial level or because in the absence of live virus the v-SRBC binding sites were perhaps more evenly distributed among the epithelial cells. The increase in secretory and serum Ab titers, however, was more pronounced in the volunteers than in patients. As shown in Table 2 the correlation between increases in the number of Ab-carrying cells and serum Ab levels was high in both patients and volunteers (r = 0.46 and r = 0.56 respectively). In patients, however, the number of Ab-carrying cells did not correlate with Ab levels in washings (r = 0.14), while in immunized volunteers these parameters correlated rather well (r = 0.70) with each other.

TABLE 2

Degree of correlation between kinetics of antibody appearance in serum, nasal washings and at the surface of respiratory epithelial cells[a]

Correlation tested	Correlation coefficient (r)	
	Patients	Volunteers
Circulating Ab vs. cell-bound Ab	0.46	0.53
Circulating Ab vs. washing Ab	0.89	n.d.
Washing Ab vs. cell-bound Ab	0.14	0.70

[a] Changes in antibody levels between first and second time point (see Table 1) were tested for correlation by Pearson's rank analysis.

DISCUSSION

Our results demonstrate that measurement of virus-coated SRBC adsorption to cell surfaces is a suitable technique for the demonstration of specific anti-influenza virus Ab bound to respiratory epithelial cells during and late after an influenza A(H1N1) infection. Evidence in support of the specificity of the technique is that haemadsorption to epithelial cells did not occur when either virus-free SRBC or A(H3N2) SRBC conjugates were used as a probe. Another control was that epithelial cells from the respiratory tract of normal rats failed to adsorb v-SRBC (data not shown). Similar evidence for specificity of cell-bound Ab has been reported in the literature [3,7]. Furthermore, the Ab found on the surface of epithelial cells have been characterized as sIgA [5]. Thus, one may assume that v-SRBC attachment to respiratory epithelial cells is due to the presence of specific Ab of the sIgA class.

It is well known that secretory immunoglobulins are highly adhesive in general [4]. Therefore, their presence may reflect simple adsorption of immunoglobulins present in secretions. On the other hand, epithelial cells might also play an active role in the secretion of immunoglobulins, and in this case the incorporation of Ab in the apical cell membrane may be part of this active process. Neither of these alternatives has as yet been explored experimentally. A methodological basis for such exploration is provided by the technique used in the present study. As demonstrated by our results, the technique allows coordinative determinations of cell-bound and cell-free Ab in the respiratory mucosa. Especially in immunized volunteers, application of the technique allows monitoring of the three components of the humoral arm of the immune system in anti-influenza immune response: serum Ab, secretory Ab, and Ab-carrying cells.

In the case of cells collected from infected individuals, part of the cell-bound Ab may be blocked by virus particles and may thus be unavailable for the SRBC conjugate. This may explain why in our study the lowest number of positive cells occurred at the time of highest reproductive activity of the virus (2–3 days). Also, the highest number of positive cells was noted when the virus had already been eliminated. In the case of cells collected from immunized volunteers, virus blocking of cell-bound Ab is an improbable alternative. Accordingly, a good temporal correlation ($r = 0.70$) was seen between the numbers of Ab-carrying cells and the content of Ab in wash fluids. This correlation was not seen in the group of patients.

The assumed blocking of surface Ab in the presence of replicating virus may also affect the correct evaluation of the number of v-SRBC per positive cell. Thus, in infected patients this number increased 2-fold at the second time point. In contrast, in immunized volunteers it did not significantly differ between both time points, although the number of Ab-positive cells increased 6-fold.

The discrepancy between the levels of solubilized Ab and the degree of Ab fixation to epithelial cells demonstrated in infected individuals may be due to several effects of the replicating virus: blocking of Ab in the fluids or on the cell surfaces, lysis of cells, etc. Therefore, it may be difficult to determine the total amount as well as the mode

of attachment of Ab during the course of an infection. Nevertheless, the very low correlation found in this situation ($r = 0.14$) as compared to the well correlating kinetics of washing Ab levels and Ab-carrying cells in volunteers ($r = 0.70$) suggests destruction of the local immune defense by the infectious virus.

The number of Ab-carrying cells was higher in convalescents in the late post-epidemic period than in the initial number in patients (1.59 vs. 0.67; $P < 0.05$). This indicates that the presence of cell-bound Ab is a useful indicator, in addition to the presence of serum Ab, for assessing previous influenza virus infection. The presence of secretory Ab in washings is an insufficient parameter in this respect.

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