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SECTION 2

MECHANISMS OF IMMUNITY TO INFLUENZA IN EXPERIMENTAL ANIMALS

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The role of humoral immunity in host defence against influenza A infection in mice

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Summary

The respective roles of cell-mediated immunity and humoral immunity in host defence were investigated in mice infected with influenza A/PR8 virus. Transferred immune spleen cells were shown to provide full protection only when they were actually secreting antibody. Serum antibody transferred in 'physiological' amounts was found to be protective in immunologically intact or in immunosuppressed animals. The specificity of the transferred antibody was shown to be critical, since antibody to internal components of the virus was inefficient while antibody to haemagglutinin, especially that to the strain-specific determinants of the haemagglutinin molecule, was highly efficient.

Introduction

Despite the extensive knowledge that has accumulated on the biochemistry, serology and epidemiology of the influenza A virus, surprisingly little is known of the mechanisms of host defence against influenza infection. In man, the epidemiological findings are conflicting, and the evidence available is mainly relevant to serological correlates of immunity rather than to the mechanisms involved in protection (Stuart-Harris, 1972). The experimental animal lends itself to investigation of the important question of the respective role of humoral versus cell-mediated immunity in protection against influenza infection. Using an avian model, a study involving bursectomy and thymectomy suggested that cell-mediated immunity is less important than humoral immunity in recovery from infection (Portnoy, Bloom and Merigan, 1973). In mice, it has been shown that passive transfer of post-infection immune serum can protect against influenza infection (Loosli, Hamre and Berlin, 1953) and that transfer of anti-neuraminidase antibody is also protective (Schulman, Khakpour and Kilbourne, 1968). However, unequivocal evidence for a humoral mechanism for resistance would be provided by the observation that serum antibody transferred to susceptible individuals in concentrations found in animals immunized by natural infection can passively protect both normal and immunodepressed recipients. Using this approach in either normal, cyclophosphamide-treated, or thymus-deprived animals, we have shown that physiological amounts of specific antibodies, especially those directed against haemagglutinin, protect in the absence of an active host response (Virelizier, 1975). The essential role of antibody to external antigens of the virus is further underlined by our finding that mice actively or passively immunized against internal components of influenza A virus are not significantly protected (Oxford and Schild, 1976).

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Materials and methods

Mice

Adult CBA mice 6–8 weeks old were used. Thymus-deprived, irradiated, and bone marrow reconstituted animals (TXBM) were obtained as described previously (Virelizier, 1975). Normal control mice (T+) were untreated animals from the same batch.

Cyclophosphamide (CY) treatment

Mice were injected intraperitoneally (i.p.) with a sublethal dose (300 mg/kg) of cyclophosphamide (Endoxana, Ward Blenkinsop, London). The techniques used for transfer of spleen cells, detection of viruses in mouse organs, and intranasal inoculation, have been described previously (Virelizier, 1975).

Measurement of antibody

This was done by the single radial immunodiffusion method as described by Schild, Henry-Aymard and Pereira (1972). A purified concentrated preparation of intact A/PR8/34 (H_0N_1) virus was mixed in agarose at a final concentration of 0·15 mg of viral proteins/ml. Antibody potency was measured by the annulus area (mm^2) of the zone of opalescence appearing around 2 mm diameter wells containing 5 μl volumes of test antiserum.

Viruses

The virus stains employed, the purification of their haemagglutinins and preparation of anti-influenza virus or anti-haemagglutinin antisera have been described previously (Virelizier, 1975).

Preparation of matrix protein (M) and nucleoprotein (NP) antigens

Concentrates of influenza virus (15 mg protein/ml) were disrupted by the addition of 1% final concentration of sodium sarkosyl detergent (Ciba-Geigy NL97) in 0·05 mol/l sodium phosphate buffer pH 6·6 at room temperature. Approximately 30 μl of solubilized virus was streaked on to each of several cellulose strips (Oxoid) and electrophoresis carried out at 180 V for 6 hr. A side piece of each strip was removed, stained for protein using procean brilliant blue (Laver, 1964) and matched up to the original strip. Under these conditions the M protein was contained in a well demarcated band moving most rapidly to the anode. The protein from ten strips was eluted at 4°C overnight into 5 ml of PBS, dialysed against PBS for 2 days at 4°C to remove detergent and used as antigen. Preparations were analysed by SDS polyacrylamide gel electrophoresis to verify absence of other virus polypeptides (Oxford, 1973). Nucleoprotein antigen was contained in a protein band moving more slowly towards the anode behind the MP band under the above conditions and was similarly located, eluted from the strips, dialysed and stored at −70°C until used as antigen (Oxford and Schild, 1976).

Results

Transfer of immune spleen cells

Table 1 shows that transfer of spleen cells from immune resistant donors into susceptible gnotobiotic

TABLE 1. Mortality after intranasal inoculation with a lethal dose of A/PR8 virus * in mice actively or passively immunized before infection

Immune status before infection	Anti-/A/PR8 antibody titres 4 hr before infection**	Proportion of dead mice 15 days after infection	Mean day of death
Untreated	0	30/30	7·1
Survivors of a previous sublethal A/PR/8 infection†	4·9	0/12	-
Recipients of spleen cells from survivors of a previous sublethal A/PR8 infection‡	0	10/13	9·3
Recipients of immune spleen cells also injected with H_0 haemagglutinin§	19·0	0/7	–
No cell transfer Injection of H_0 haemagglutinin #	0	6/6	9·7

* $10^{2.2}$ EID_{50}/mouse.

† Mice were infected i.n. 2 months previously with a non-lethal dose (10^{-4} dilution) of A/PR8 virus.

‡ 10^8 viable cells collected from spleens of immune donors (2 months after recovery from a non-lethal infection) were injected i.p. into recipients 8 days before infection.

§ Recipients of the same pool of spleen cells given to preceding group were injected i.p. with 10 μg H_0, 24 hr after cell transfer, i.e. 7 days before infection.

10 μg H_0 were injected i.p. 7 days before infection in control (gnotobiotic) animals.

** A pool of sera from each group was analysed by SRDT in immunoplates containing A/PR8 virus. Titres are expressed in mm^2 of zone area.

TABLE 2. Protection provided by various types of anti-influenza sera transferred before infection in gnotobiotic recipients infected with a lethal dose of A/PR8 virus (10^{-2} dilution): results of a representative experiment

Antiserum administered T = −4 hr*	Antibody titres in recipients T = −2 hr†	Proportion of dead mice 15 days after infection
Normal rabbit serum	0	20/20
Rabbit anti-H_3 (unrelated HA)	0	6/6
Rabbit anti-H_2N_1 (homologous neuraminidase)	5·4	3/6
Rabbit anti-H_0 (homologous HA)	2·0	0/6
Rabbit anti-PR8 (homologous virus)	2·2	0/6

* Mice were injected i.p. with 0·1 ml of undiluted rabbit antisera raised against either purified HAs or viruses (see Materials) 4 hr before infection.

† A pool of sera from each group was assayed in SRDT immunoplates containing A/PR8 virus. Titres are expressed in mm^2 of zone area.

syngeneic recipients was not followed by spontaneous secretion of antibody in the host as detected by single radial diffusion tests. Intranasal infection with a lethal dose of homologous A/PR8 virus showed that protection of the recipients was poor and incomplete. In contrast, when the same number of transferred spleen cells were 'boosted' in the recipients with a small dose of A/PR8 haemagglutinin (H_0), insufficient by itself to provide either an antibody response or protection, an abundant secondary antibody response occurred and all recipients survived.

Protection in recipients of antibody to external influenza antigens

As shown in Table 2, transfer of antibody to H_3 (A/Hong Kong/68 haemagglutinin with no antigenic relationship to H_0) did not provide any protection against a lethal infection with A/PR8 virus. In contrast, transfer of antibody to H_0 (the homologous haemagglutinin) or to N_1 (the homologous neuraminidase) protected the recipients. However, more anti-neuraminidase antibody was needed to obtain a significant protection, suggesting a better protective efficiency by anti-haemagglutinin antibody.

Absence of protection in mice actively or passively immunized against M or NP antigens

As shown in Table 3, transfer of potent rabbit antisera to MP or NP, the two major internal components of influenza A viruses, did not protect against an A/PR8 infection. In addition, transfer of a mixture of anti-M and anti-NP antibody did not provide any detectable protection. Moreover, active immunization with these two structural antigens also failed to confer protection to a lethal challenge with A/PR8 virus, as shown in Table 4.

TABLE 3. Effect of passive antibody on A/PR8 infection in mice

Pre-treatment hyperimmune rabbit sera to*	Proportion of dead mice 15 days after infection	Mean day of death
None	6/10	8·5
HA (PR8)	0/10	–
MP	9/10	11·0
NP	13/19	9·6

* Mice were injected with 0·1 μl of undiluted rabbit serum raised against purified HA, MP, or NP antigens.

TABLE 4. Effect of active immunization with structural antigens on A/PR8 in mice

Immunization	Proportion of dead mice 15 days after infection	Mean day of death
None	23/25	6·2
B/LEE virus	16/19	8·5
A/PR8 virus	0/18	–
Matrix protein	25/31	6·9
Nucleoprotein	26/38	7·4

Animals were immunized with two weekly doses of 20 μg virus protein and challenged with virus 10 days after the final immunization dose.

Protection of cyclophosphamide-treated mice by transferred antibodies

Table 5 shows that mice receiving CY 18 hr after infection with a dose of A/PR8 virus, which was non-lethal in normal untreated mice, did not produce detectable antibody 7 days after infection and died. Other groups of mice were injected, 24 hr after infection, with a dose of rabbit antiserum found empirically to provide in recipients titres of serum antibodies similar to those observed in intact animals 7 days after infection. It was shown that while recipients of anti-A/PR8 and anti-A/FM1 virus (whole

TABLE 5. Protection of immunodepressed mice against lethal A/PR8 infection by various anti-influenza sera

Intranasal* inoculation T = 0 hr	Cyclophosphamide* administration T = 18 hr	Antiserum† administered T = 24 hr	Proportion of dead mice at T = 15 days	Mean day of death	Anti-PR8 antibody titres‡	
					T = 7 days	T = 21 days
Saline	+	0	0/6	–	0	0
A/PR8 10^{-4}	0	0	0/6	–	1·4 (0·3–2·6)	6·3 (2·6–8·2)
A/PR8 10^{-4}	+	Normal rabbit serum	6/6	11·5	0	–
A/PR8 10^{-4}	+	Rabbit anti-A/PR8	0/6	–	0·8 (0·3–1·0)	NT
A/PR8 10^{-4}	+	Rabbit anti-A/FMI	4/6	10·0	2·2 (0·3–2·8)	NT
A/PR8 10^{-4}	+	Rabbit anti-H_0	0/6	–	1·5 (1·3–3·4)	0·4 (0–0·7
A/PR8 10^{-4}	+	Rabbit anti-HI	6/6	11·7	2·2 (1·8–3·9)	–

* Virus and cyclophosphamide treatment as described in Methods.
† Mice were injected i.p. with 0·1 ml of a dilution of anti-influenza sera found to provide serum antibody titres similar to those secreted by non-CY-treated animals.
‡ Sera were tested individually in single-radial-immunoplates containing A/PR8 virus. Titres are expressed in mm^2. Range of titres are indicated between brackets.

TABLE 6. Protection of thymus-deprived (TXBM) mice against lethal A/PR8 infection by anti-H_0 antiserum

Type of mice	Dilution of A/PR8 virus inoculated IN T = 0 hr	Anti-H_0 serum* administration T = 24 hr	Proportion of A/PR8 virus† recovery at		Proportion of spontaneous death
			T = 6 days in lungs	In brain	
T+	10^{-2}	0	NT	NT	6/6
TXBM	10^{-2}	+	NT	NT	0/6
T+	10^{-2}	0	6/6	2/6	–
TXBM	10^{-2}	0	6/6	5/6	–
TXBM	10^{-2}	+	0/6	0/6	–

* Mice in groups marked + were injected i.p. with 0·1 ml of a rabbit anti-H_0 serum titrating 100 mm^2/5 μl in SRDT.
† 20% organ suspensions were injected in fertile chick eggs. The allantoic fluids were tested for virus growth as described in Methods.

particles) were equally protected, there was a striking difference between the animals receiving anti-H_1 antibody, which all died, and the animals receiving anti-H_0 antibody, which all survived.

Protection of thymus-deprived mice by transferred anti-H_0 antibody

Figure 1 shows that thymus deprivation potentiates influenza A infection and decreases serum antibody levels, especially in mice (approximately 50% in repeated experiments) which died before day 21. TXBM mice remaining alive at day 21 after infection still had detectable influenza virus in the brain and lungs, at a time when organ suspensions from infected control mice were negative. Transfer of antibody to H_0 24 hr after infection in TXBM mice was effective in protecting against death and in clearing the virus in both brain and lung, as shown in Table 6.

Discussion

The results reported in the present communication suggest that humoral immunity is more important than cellular immunity in protection against influenza A virus infection in mice. Recipients of immune spleen cells were very poorly protected against a lethal challenge with A/PR8 virus. This was not due to inefficient transfer of immunity, since the presence of both thymus-derived (T) and bone-marrow derived (B) memory cells in the recipients was confirmed by the typical secondary antibody response obtained after 'boosting' the host with a dose of purified H_0 which was not immunogenic in virgin animals. Such boosted recipients were shown

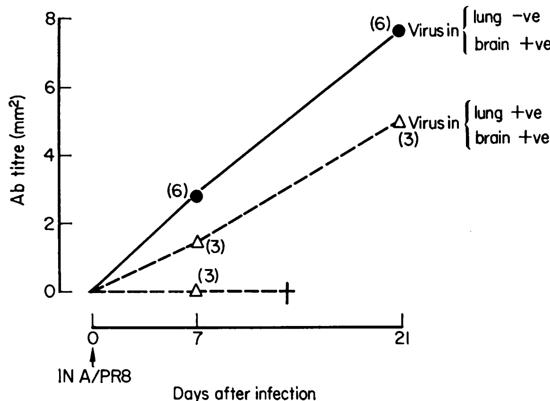

FIG. 1. Course of infection in thymus-deprived mice. Normal (T+, ●—●) and thymus-deprived (TXBM, △ - - △) mice were inoculated intranasally with 50 μl of a 10^{-4} dilution of A/PR8 virus ($10^{0.25}$ EID_{50}). Sera were tested individually by SRDT in immunoplates containing A/PR8 virus, and the arithmetic mean of zone areas for each group calculated. The number of mice tested is given in brackets. Results from TXBM animals with no detectable antibody 7 days after infection are plotted separately. Presence of A/PR8 virus in lungs and brains was assessed as described in Methods.

to be fully protected, indicating that immune spleen cells are fully protective only when they secrete antibody. Conversely, it has been found that CY treatment prevents the anti-influenza antibody response and potentiates influenza infection. This finding apparently conflicts with reports that CY (Singer, Noguchi and Kirschstein, 1972) or anti-lymphocytic serum treatment (Suzuki, Ohya and Ishida, 1974) may lead to a less severe disease in influenza-infected mice. However, experimental conditions may be critical, and immunosuppression may decrease antibody production in some systems, and decrease the cellular recruitment, thereby minimizing the resultant harmful lung consolidation in others. Indeed a recent publication (Hurd and Heath, 1975) reconciles these different data by showing that CY increases the mortality of mice infected with low concentrations of influenza virus, but delays the time of death in mice infected with high concentrations of the same variant.

The finding that transfer of serum antibody, resulting in serum titres in the recipients comparable to those found in mice which will later recover from infection, can protect both normal and immunosuppressed mice, strongly suggests that humoral immunity plays a major role in protection under natural conditions. Since the analysis of the authors' hyperimmune rabbit antisera by rate centrifugation in sucrose gradients showed that they contained exclusively 7S anti-influenza immunoglobulins, it can be assumed that IgG antibody was in fact protective in their method. While the role of respiratory IgA remains speculative, the results suggest that IgG molecules are efficient against a pulmonary infection. This is in agreement with reports that in ferrets (Shore, Potter and McLaren, 1972) as well as in horses (Rouse and Ditchfield, 1970) the nasal antibody to influenza virus appears to be a 7S IgG protein.

In our study, the specificity of the transferred antibody was apparently critical. Antibody to internal components of the influenza virus (M and NP) appeared to have no relevance to protection in our system, even when given in large amounts. This appears to conflict with the finding (Schulman and Kilbourne, 1965; Werner, 1966; Kurimura, Hirano and Okuno, 1973; Oxford and Schild, unpublished observations) that intratypic cross-immunity can be found in mice previously infected with A/HK/1/68 (H_3N_2) and challenged with A/PR8/34 (H_0N_1). However, in post-infection sera, antibodies with as yet unrecognized specificity may contribute to protection, as suggested by other authors (Masurel, Baars and Frankena, 1973; Sweet, Stephen and Smith, 1974). In contrast to the absence of protection with antibody to internal structural antigens, transfer of antibody to external components proved highly effective. Anti-neuraminidase antibody appeared less effective, however, than anti-haemagglutinin antibody. In a previous communication (Virelizier *et al.*, 1974) evidence was provided that H_0 and H_1 haemagglutinins share common antigenic determinants eliciting cross-reacting (CR) antibodies, but differ in their strain-specific determinants eliciting a strain-specific population of antibodies (called SO in the case of an anti-H_0 response). Our results suggest that SO antibodies have a much better protective efficiency than CR antibodies. This finding is in accordance with the relative inefficacy in man of influenza vaccines containing previously prevalent influenza virus strains rather than the current prevalent virus.

These findings do not rule out the possibility that cell-mediated immunity plays an active role in host defence against influenza virus infection under natural conditions. Indeed, it has previously been reported that antibody formation to influenza haemagglutinin is impaired in thymus-deprived mice, the secretion of strain-specific antibody being especially thymus-dependent (Virelizier *et al.*, 1974). Moreover, it has been shown that T cells regulate the production of anti-haemagglutinin antibodies by either enhancing or suppressing their secretion by B memory cells (Virelizier, Allison and Schild, 1974). The present study confirms that infected TXBM animals indeed had a decreased antibody response and a higher mortality than the controls. This suggests that T lymphocytes may have an indirect but essential role

in host defences against influenza infection by enhancing the secretion of protective strain-specific anti-haemagglutinin antibodies.

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