**The effect of long-term exposure
to cigarette smoke on the height and specificity of the
secondary immune response to influenza virus
in a murine model system**

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(Received 6 October 1978)

SUMMARY

The effect of long-term exposure to cigarette smoke on the height and specificity of the secondary humoral immune response to influenza was investigated in a murine model system. It was shown that if mice were pre-immunized with a sub-lethal infection of influenza virus and then exposed to cigarette smoke daily for 36 weeks, they were able to mount a secondary immune response of normal height on subsequent challenge with the homologous virus strain. The response, however, was less specific than that elicited in control mice, with high titres of cross-reacting antibody by haemagglutination-inhibition to the following strain in the same antigenic series. Recall of antibody to the previous strain in the antigenic series was not observed in either control or smoke-exposed animals. These results serve to correct an earlier discrepancy between the murine system and human studies in which the response to influenza infection in mice was depressed by prolonged exposure to cigarette smoke, whereas in man the response of smokers did not differ significantly from that of non-smokers. This apparent discrepancy had been caused by a lack of previous experience of influenza in the mice, which had therefore mounted a primary response, compared with the secondary response observed in the human studies.

INTRODUCTION

Cigarette smokers have been shown to be more susceptible to infection with epidemic influenza than non-smokers (Finklea, Sandifer & Smith, 1969; Mackenzie, Mackenzie & Holt, 1976), providing they possessed little or no pre-epidemic haemagglutination-inhibiting (HI) antibody (HI titres of < 12) (Mackenzie *et al.* 1976). Humoral HI antibody titres to influenza were significantly increased among smokers who remained well and minimally increased among smokers who were sick, compared with those of non-smokers (Finklea *et al.* 1971), which suggested that smokers were also more susceptible to subclinical infections. There was no difference among smokers in their immunological response to vaccination with killed whole virus (Finklea *et al.* 1971) or subunit (Mackenzie *et al.* 1976) influenza vaccines, but the longevity of the immune response to the subunit

vaccine was severely depressed 50 weeks after vaccination in smokers who had possessed little or no immunity (HI titres of < 12) before vaccination (Mackenzie *et al.* 1976). A significantly higher proportion of smokers, however, sero-converted after receiving a live attenuated influenza A virus vaccine than their non-smoking counterparts, but the longevity of the immune responses was similar in the two groups (Mackenzie *et al.* 1976).

A murine model system was established to explore the effect of cigarette smoke on infectious and neoplastic diseases (Holt, Keast & Mackenzie, 1978). It was found that long-term exposure of mice to cigarette smoke severely depressed both the cell-mediated immune response within the respiratory tract (Thomas, Holt & Keast, 1973*a*) and the primary humoral immune response to intra-tracheal stimulation with sheep erythrocytes (Thomas, Holt & Keast, 1973*b*). Prolonged exposure of mice to cigarette smoke was also shown to depress the humoral immune response following intranasal infection with influenza A virus, and to decrease the frequency of sero-conversion (Mackenzie, 1976). This depressed HI antibody response in mice after long-term exposure to cigarette smoke was not observed in the human studies. Indeed, the immune response of human smokers to epidemic influenza or to influenza vaccination was not significantly different from that of non-smokers. The probable explanation for the apparent discrepancy is that the mice had had no previous exposure to influenza whereas the human smokers would have had frequent prior experiences to earlier strains, and would therefore have responded, in part, with a secondary response to the cross-reacting determinants of the haemagglutination.

Thus the purpose of this study was to determine the effect of exposure to cigarette smoke on the level and specificity of the secondary immune response to influenza in a murine system.

MATERIALS AND METHODS

Virus strains

Influenza virus strains A/MEL (H0N1), A/CKS (H0N1) and A/BEL (H0N1) were employed in this study. Virus stocks were grown in the allantoic cavity of 11-day embryonated eggs. Allantoic fluids were harvested after 40 h incubation, clarified by centrifugation at 800 *g* for 20 min, and stored in 1.0 ml volumes at -70°C .

Mouse strain and smoking schedule

C3H/HeJ inbred female mice were obtained from the Small Animal Breeding Unit, University of Western Australia. The mice were exposed to fresh cigarette smoke in a Hamburg II (Heinrich Borgwaldt, FRG) small animal smoking machine set to deliver a mixture of smoke:air (1:7) at a puff volume of 35 ml. This corresponded, by body weight, to 20–30 cigarettes per day of human consumption (Chalmer, Holt & Keast, 1975). The mice were exposed daily for approximately 8 min over a period of 36 weeks. Age-matched control mice were kept over the same time period.

Mouse inoculation

Lightly etherized mice were inoculated by the intranasal route with 25 μ l virus diluted in PBS to give a dose of 0.1 LD 50. Control mice were inoculated similarly with diluent only.

Collection of specimens and serological assay

Blood specimens were collected from the retro-orbital venous plexus and the serum separated by centrifugation. The serum samples were treated with cholera filtrate to destroy non-specific inhibitors, and titrated for antibody directed against the viral haemagglutinin by haemagglutination-inhibition (HI). Four haemagglutinating (HA) units of virus were incubated with serial twofold serum dilutions in citrate buffer (pH 7.2) at 4 °C overnight, before the addition of a 0.5% suspension of fowl erythrocytes. Titres were expressed as the reciprocals of the dilution at which haemagglutination was completely inhibited. The sensitivity of the secondary immune response to 2-mercaptoethanol was determined by diluting the serum samples 1 in 10 with citrate buffer pH 7.2 containing 0.1 M 2-mercaptoethanol, and incubating the samples for 1 h at room temperature. The treated samples were assayed for HI antibody as above, using a diluent containing 0.01 M 2-mercaptoethanol.

RESULTS

Effect of long-term exposure to cigarette smoke on the secondary immune response

Eight-week-old C3H/HeJ mice were inoculated intranasally with 0.1 LD 50 of influenza strain A/MEL. A blood specimen was drawn from the retro-orbital venous plexus 14 days after infection to determine the primary immune response. The mice were then exposed daily to cigarette smoke for 36 weeks and, immediately on termination of the smoking schedule, they were challenged with a similar sub-lethal intranasal inoculation of the homologous virus. Three weeks later serum samples were collected, and assayed individually for HI antibody titres. The geometric mean HI antibody titres of the primary and secondary responses in the smoke-exposed and unexposed control mice are shown in Table 1. Long-term exposure to cigarette smoke did not affect the ability of the mice to elicit a normal secondary immune response, and although the geometric mean HI antibody titre was slightly lower than that observed for the control animals, the difference was not significant. Confirmation that the antibody titres were due to a secondary immune response was obtained by treatment of the serum samples with 2-mercaptoethanol. All samples obtained after challenge were found to be resistant to 2-mercaptoethanol, and no reduction in the geometric mean HI antibody titres was observed.

Table 1. *The effect of long-term exposure to cigarette smoke on the secondary humoral immune response in mice pre-immunized with influenza*

	Geometric mean HI antibody titres*	
	Exposed to cigarette smoke	Control
Pre-immunization	< 6	< 6
14 days post-infection	24	24
21 days after challenge†	180	250

* 15 mice per group.

† Mice were challenged with 0.1 LD 50 of the homologous virus after daily exposure to cigarette smoke for 36 weeks.

Effect of long-term exposure to cigarette smoke on the specificity of the secondary immune response

Three different strains of influenza A virus were employed to examine the effect of cigarette smoke on the specificity of the secondary immune response. The three strains, A/MEL, A/CKS and A/BEL, were different antigenic drift isolates from the H0N1 antigenic series having been isolated from human cases in 1935, 1941 and 1942 respectively.

Mice, which had been pre-immunized with 0.1 LD 50 of A/MEL by the intra-nasal route before exposure to cigarette smoke, were challenged with a similar dose of either A/MEL or A/CKS immediately on termination of the 36-week smoking schedule. Three weeks later they were each given a lethal injection of sodium pentobarbitol (Abbot Laboratories, Sydney) intraperitoneally, and exsanguinated from the axilla. The sera were collected and assayed for HI antibody using A/MEL, A/CKS and A/BEL as antigens in the HI assay. The results of the assays as geometric mean HI titres are shown in Table 2.

It was found that after challenge with the homologous virus, A/MEL, the control animals mounted a secondary immune response that was specific to MEL with little or no stimulation of the cross-reacting antigenic determinants on the haemagglutinins of CKS or BEL. The response of smoke-exposed mice, however, was less specific with significantly increased titres of cross-reacting antibody to the haemagglutinin of CKS, and to a less extent, of BEL. Similar results were observed if the mice were challenged with A/CKS. The response in control mice was specific to CKS, whereas the response in smoke-exposed mice was less specific with a high cross-reacting geometric mean titre to BEL. This latter result, however, was surprising in that neither control nor smoke-exposed animals challenged with CKS were found to exhibit an antigenic recall to MEL. Thus no evidence was observed of original antigenic sin.

Table 2. *Effect of long-term exposure to cigarette smoke on the specificity of the secondary immune response in mice pre-immunized with influenza virus strain MEL*

Challenge virus strain†	Influenza strain in HI assay	Geometric mean HI antibody titres*	
		Exposed to cigarette smoke	Control
MEL	MEL	180	250
MEL	CKS	150	45
MEL	BEL	50	30
CKS	MEL	65	45
CKS	CKS	180	150
CKS	BEL	205	75

* Ten mice per group.

† Mice were pre-immunized with influenza strain A/MEL and challenged with 0.1 LD₅₀ of A/MEL or A/CKS after daily exposure to cigarette smoke for 36 weeks.

DISCUSSION

Prolonged exposure of mice to cigarette smoke had previously been shown to depress the primary immune response following intranasal infection with influenza A virus (Mackenzie, 1976). Immuno-suppression, however, had not been observed in human studies: the immunological responses of smokers to killed (Finklea *et al.* 1971; Mackenzie *et al.* 1976) or live (Mackenzie *et al.* 1976) influenza vaccines did not differ significantly from those of non-smokers, regardless of whether or not they possessed pre-existing HI antibody (Mackenzie *et al.* 1976). The major discrepancy between the murine model system and the human studies, therefore, has been resolved. The results suggest that in the earlier study the depressed immune response in mice was due to their lack of prior exposure to influenza, whereas human smokers would have had frequent exposure to influenza strains both before and after commencing the smoking habit.

An apparent lack of antibody specificity in the secondary immune response was displayed by smoke-exposed animals in the HI assay, compared to their non-smoking counterparts. The reasons for this are unknown. Cigarette smoking, however, has been shown to have profound effects on body defence mechanisms in man and in laboratory animals and to induce a number of changes in immunological function (Holt, Thomas & Keast, 1974; Holt & Keast, 1977). Long-term exposure of experimental animals to cigarette-smoke was found to lead to increased numbers of pulmonary alveolar macrophages (Holt & Keast, 1973; Rylander, 1974) which exhibited reduced bactericidal activity (Rylander, 1971), and to leukopenia distal to the respiratory tract (Holt *et al.* 1976). Phytohaemagglutinin reactivity of lymphocytes from the spleen, peripheral blood and the regional lymph nodes draining the lungs were depressed (Thomas *et al.* 1973*a*), and although the antibody responses to a B-lymphocyte dependent antigen were normal, the responses to a T-lymphocyte dependent antigen were reduced to 25% of control

levels (Thomas, Holt & Keast, 1975). These findings suggest that antigen-processing by alveolar macrophages might also be substantially affected. Moreover, the immune response to influenza has been shown to be a B-lymphocyte response in mice, but modulated by T-lymphocytes (Virelizier, 1975; Virelizier, Allison & Schild, 1974). Thus the changes induced by long-term exposure to cigarette smoke on immune function, particularly those mediated by T-lymphocytes, may result in an alteration of the specificity of the immune response.

Since similar effects on immune function have been reported from human studies (Holt & Keast, 1977), it would be interesting to determine the specificity of the antibody responses in human smokers following both vaccination and exposure to epidemic influenza.

No explanation is readily available for the lack of antigenic recall to MEL by smoke-exposed and control mice pre-immunized with MEL and subsequently challenged with CKS.

This study was supported by research grants from the Australian Tobacco Research Foundation and the National Health and Medical Research Council of Australia.

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