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INFLUENZA VIRUS DEPRESSES THE PFC RESPONSE OF MICE BY AFFECTING T-CELL FUNCTION

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Summary. — Five influenza virus strains of type A and one strain of type B were used to elicit suppressive effect on the formation of antibody producing cells in response to sheep red blood cells (SRBC) both in vivo and in vitro. Not only live virus but also the formalin inactivated one exerted immunosuppression (IS). Although intranasal infection only could cause disease in mice, it exerted no IS which, in contrast, occurred after intraperitoneal or intravenous injection of the virus. In nude mice these effects were not seen, but they could be elicited when transferred with normal T cells. The antibody response to T cell independent antigen such as DNP was normal. Thus, it was proved that influenza virus induced T cell dependent immunodeficiency. However, T cell enriched population from infected mice when cultured with normal spleen cells could not suppress their PFC response in vitro. This suggested that suppressor T cells were not required for the immunodeficiency caused by influenza virus.

Key words: immunodeficiency; influenza virus (live and inactivated); mice; T cell

Introduction

A large variety of infectious agents, of viral (Bendinelli, 1981; Makarov and Chevelev, 1984), bacterial (Poindexter and Schlievert, 1986) or protozoan origin (Cunningham *et al.*, 1980) have been reported to be accompanied by immunosuppression (IS). This is clearly important in terms of the susceptibility of the host to secondary infections, as seen in acquired immune deficiency syndrome (Armstrong, 1984).

During the acute phase of influenza infection, the development of bacterial superinfection is a major cause of severe complications or death (Couch, 1981). It is well-known that during the acute stages of measles infection the positive tuberculin skin test becomes temporarily negative. Occasionally, the same may occur in influenza infection (Hooren, 1976). Many immunological functions might be impaired in man, i.e. cell-mediated immunity (Dolin *et al.*, 1977), in vitro reactivity of lymphocytes and macrophages (Roberts *et al.*, 1978) and chemotaxis of monocytes (Pike *et al.*, 1977). In experimental

influenza virus infection of laboratory animals, suppression of delayed type hypersensitivity (Massanari, 1979), dysfunction of polymorphonuclear leukocytes (Abramson *et al.*, 1982) and macrophages (Kleinerman *et al.*, 1976) or suppression of humoral antibody response to antigens unrelated to influenza virus (Kavetzky *et al.*, 1977; Nizamov and Shuratov, 1980; Kiseleva *et al.*, 1982) have been reported.

We studied the mechanism of the suppressive effect of influenza virus on the formation of the antibody producing cells in response to sheep red blood cells in mice. The main aim of the present study was to determine which immunologically active cells were responsible for IS caused by influenza virus. We assumed that the deficiency in T helper cells might be of principal importance.

Materials and Methods

Virus strains. Five strains of influenza virus type A: A/PR/8/34(H1N1), A/WSN/33(H1N1), A/MEL/35(H1N1), A/CAM/46(H1N1), A/Hong Kong/8/68(H3N2), and strain B/Lee/40 were used. The viruses were grown in embryonated hen eggs. The chorioallantoic fluid contained 2^9 to 2^{12} HAU/50 μl. Virus was inoculated by intraperitoneal (i.p.), intravenous (i.v.) or intranasal (i.n.) routes. Strains PR/8, CAM and MEL caused clinical symptoms and death in Balb/c mice after i.n. inoculation of 0.1 HAU. On the other hand, WSN, Hong Kong and B/Lee never caused disease or death upon i.n. infection. After i.p. injection of any strain neither clinical symptoms nor death occurred.

Haemagglutinin (HA) of PR/8 virus, obtained by ultracentrifugation in caesium sulphate density gradient was the gift of Dr. Akira Ishihama (Institute for Virus Research, Kyoto University). It was depleted of contaminating NP-40 by passage through Biobeads column. After this treatment thes HA sample contained 2^{12} HAU/50 μl.

Virus inactivation. Four strains of type A (PR/8, WSN, CAM and MEL) were inactivated by incubation with 0.02% formalin at 4 °C for 7 days. The inactivated samples contained 2^7 to 2^8 HAU/50 μl and were noninfectious for eggs.

Animals. Balb/c (SPF), and congenitally athymic Balb/c mice (nu/nu) were purchased from the Shizuoka Experimental Animal Cooperation. Female mice at 7 to 11 weeks of age were used in all experiments. They were caged in an independent isolator system.

Antigens. Sheep red blood cells (SRBC) in Alsever's solution were purchased from Nippon Bio-Test Laboratories Inc., Tokyo and washed three times with phosphate buffered saline (PBS) before use. They were resuspended to 1% or 10% in PBS just before inoculation. Mice were immunized by i.p. inoculation of 0.1 ml of this preparation. 1% solution contained about 2×10^7 cells in 0.1 ml. When nude mice were used, 4×10^8 SRBC were administered i.p. DNP-lys-Dextran T 2000 (2.4 dinitrophenyl-lysyl derivate of Dextran T 2000) was prepared by a modification of the method described for DNP-lysl-Ficoll by R. Sharon *et al.* (1975). DNP-l-lysine was obtained from Tokyo Kasei Co. Ltd. Dextran T 2000 was obtained from Pharmacia Fine Chemicals. Each mice was inoculated with 1 mg protein by i.p. route.

PFC response and assay. Quantification of anti-SRBC or anti-DNP Ig (Immunoglobulin) producing cells was made by the slide modification of Jerne's plaque forming cell (PFC) assay using a Cunningham chamber (Takahashi Giken Glass). To determine the total number of specific IgM-producing cells, the direct PFC assay was performed 6 days after immunization with SRBC, or 4 days after immunization with DNP-lys-Dextran T 2000, when each response was the highest. Specific IgG-producing cells were enumerated with the indirect PFC assay 12 days after immunization with 4×10^8 SRBC, using anti-mouse IgG rabbit sera for development. When direct PFC for DNP-lys-Dextran-T 2000 was assayed, SRBC coated with TNBS (2.4.6. Trinitrobenzene sulphonic acid, Sodium Salt, Wako pure chemical Industries, Ltd.) were used as target (Rittenberg and Pratt, 1969).

To amplify the level of PFC response, mice were injected i.v. with 4×10^7 SRBC 7 days before preparation of spleen cells. 5×10^6 spleen cells/ml of a single cell suspension were placed in 96 well

tissue culture plates (Falcon Plastics 3072) in RPMI-1640 medium containing 5×10^{-5} mol/l 2-mercaptoethanol and 20% foetal calf serum. In each well 200 µl of cell suspension was given. Each cell suspension was a pool of 3 spleens. The cultures were then stimulated with 10 µl of 1% SRBC (approximately 2×10^6 cells). Cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C. By 5 days later, IgM PFC was assayed in culture. Results were expressed as number of PFC per 10^6 viable cells and in terms of per cent suppression (%S), where %S = 1 − (PFC of infected mice spleen/PFC of normal mice spleen) × 100%. Statistical evaluation was performed by Student's t test. Data were expressed as means SEM.

Preparation of spleen cell suspension. Mouse spleen cell suspensions were prepared by passing tissue through a stainless steel mesh with RPMI-1640 medium or Eagle's MEM. The cells was washed 3 times with the medium before use.

Depletion of T cells. Spleen cell suspension at a concentration of 5×10^6 cells in 1 ml were incubated with a 1 : 4000 dilution of monoclonal anti-Thyl. 2 antibody (F7D5, Olac Ltd.,) for 30 min at room temperature and afterwards with 1 : 10 dilution of normal rabbit serum as complement source for 45 min at 37 °C. The cells were washed 3 times. By this procedure, approximately 40% of spleen cells were lysed.

T cell enrichment. T cell enriched spleen cells were prepared by passage through Sephadex G-10 (Pharmacia Fine Chemicals) columns, followed by separation on nylon wool column. By this enrichment we recovered about 20% of applied cells.

Protein A plaque assay. All Ig (immunoglobulin) secreting cells in the spleen were assayed by the Protein A plaque assay method (Gronowicz *et al.*, 1976). By the use of developing antisera of different class, splenic PFC, secreting Ig of different class, IgM, IgG and IgA were scored. 5×10^6 spleen cells were cultured with 2 µg of PWM (pokeweed mitogen, Gibco Laboratories) in 24 well tissue culture dishes (NUNC, NUNCLON). After 4 days in culture, these cells were assayed for Ig secreting cells using protein A coated SRBC as indicator cells. Briefly, protein A (Nakarai Cemicals, Ltd.) was coupled to washed SRBC with $CrCl_3$. Then these were mixed with the lymphocyte suspension and antisera to different Ig classes and complement and were applied to the Cunningham chamber. The plaques were enumerated after overnight incubation at 37 °C.

Administration of cyclophosphamide (CPA). CPA (Sigma Chemical Company) was administered i.p. either 2 days before, or at the virus inoculation day or 2 days after infection in a dose of 20 mg/kg body weight. This low dose of CPA was reported to suppress selectively precursors of suppressor T cells (Cantor *et al.*, 1978).

Transfer of serum and spleen cells from infected mice. The serum from mice injected with the virus was ultracentrifuged at 80,000 g for 3 hr to eliminate viral particles. Then 0.4 ml of the supernatant was injected i.v. to Balb/c mice. Unfractionated spleen cells from infected mice (2×10^8) were transferred by i.v. injection.

Results

Influenza virus suppressed PFC response to SRBC after i.p. and i.v. injection

Table 1 demonstrates the strong inhibiting effect of influenza virus A/CAM on IgM PFC response in mice infected by i.p. or i.v. injections. This effect was observed in mice, inoculated with the virus one day to 2 weeks before immunization with SRBC. When SRBC were inoculated 2 days before, on the same day or one month after virus inoculation, no suppressive effect was observed. A slight increase (approximately 15%) in the number of nucleus containing cells was observed in spleen 2 to 8 days after infection. No viral antigen was demonstrated by fluorescent antibody test (FAT) neither in the spleen nor in any other organ.

All strains, including type A and type B, had a strong suppressive effect on IgM PFC response to SRBC in mice inoculated by i.p. route. The IgG PFC response was assayed by indirect method. As shown in Table 1, the IgG PFC response was also suppressed in mice i.p. injected with 4096 HAU of

Table 1. Depression of PFC response of mice infected with influenza virus A/CAM by i.p. or i.v. routes

Route	dose (HAU)	days before antigen (SRBC)	IgM or IgG PFC/10^6 viable cells	% suppression
i.p	4096	30 days	IgM 693 ± 113	N.S.
		14 days	11 ± 7	98%
		7 days	7 ± 7	99%
		2 days	6 ± 16	99%
		0 day	725 ± 78	N.S.
		−2 days*	645 ± 114	N.S.
	0		716 ± 36	standard
i.v	4096	2 days	9 ± 11	99%
i.p	1000	2 days	IgM 5 ± 9	99%
	100		8 ± 10	98%
	1		11 ± 20	98%
	0.1		60 ± 33	87%
	0.01		327 ± 117	N.S.
	0		473 ± 42	standard
i.p	4096	12 days	IgG 78 ± 33	75%
	0		316 ± 34	standard

0.01 to 4096 HAU of influenzavirus A/CAM was injected i.p. or i.v. to each 3 to 5 mice. IgM PFC was performed 6 days after immunization with SRBC 2×10^7. IgG PFC assay was performed 12 days after immunization with 4×10^8 SRBC. Data are expressed as the mean SEM of each group. The t test statistics were performed.
N.S.; not significant, $P > 0.05$
% suppression = 1 − (PFC of infected mice spleen/PFC of normal mice spleen) $\times 100\%$
* SRBC was inoculated 2 days before virus infection.

A/CAM virus. Percentage of suppression was lower than that of the IgM PFC response. Table 1 demonstrates that as low as 0.1 HAU of the virus was effective for eliciting IS.

Intranasal infection with influenza virus cannot suppress the PFC response of mice to SRBC.

Table 2 shows that mice nasally infected with A/CAM virus revealed a normal response to SRBC. In mice infected 7 days before, on the same day or 2 days after the immunization with 2×10^7 SRBC a normal level of IgM PFC per 10^6 viable spleen cells was demonstrated. By indirect FAT, abundant viral antigen was found in the lungs of infected mice. FAT was negative in spleen, lymph nodes, thymus and liver.

The number of nucleated viable spleen cells decreased to about 50% of normal. Thus the number of IgM-secreting cells per spleen should have decreased by 50%. We did not consider this for IS, but for spleen atrophy only. All other strains had no suppressive effect on the IgM PFC response to SRBC in intranasally infected mice, the route which mostly resembles to natural infection. The virus dose over 1 HAU caused death as early as within 4 to 7 days, so we could not test the SRBC PFC response.

Table 2. Effect of i.n. infection of influenza virus on the PFC response

Virus strain	Days before antigen (SRBC) administration	IgM PFC/10^6 viable cells	Suppression %
none		941 ± 137	standard
A/PR/8	7 days	854 ± 223	N.S.
	4 days	896 ± 209	N.S.
	0 day	831 ± 180	N.S.
	2 days	973 ± 231	N.S.
B/Lee	4 days	952 ± 110	N.S.
A/CAM	4 days	881 ± 294	N.S.

0.1 HAU of influenzavirus A/PR/8, B/Lee or A/CAM was infected by i.n. route. PFC assay was performed 6 days after immunization with 2×10^7 SRBC. Virus was injected before 7 days, 4 days, at the same time or 2 days after immunization with antigen.
N.S.: not significant, $P > 0.05$

Inactivated virus can suppress PFC response

From the fact that intraperitoneal inoculation of the virus, which caused no evident clinical symptoms, suppressed the PFC response, we assumed that the pathogenicity or toxicity for mice might not be necessary for IS activity. Therefore, we used formalin-inactivated virus. Table 3 shows that all of 4 inactivated type A strains depressed the PFC response. This suppressive effect was as strong as live virus. Chorioallantoic fluid of infected chick embryos, used as virus samples, might contain a suppressive humoral factor other than viral particle, we separated the chorioallantoic fluid by ultracentrifugation at 80 000 × g for 3 hr to supernatant and pellet. Both samples were injected i.p. to mice and the IgM PFC response was assayed. As shown in Fig 1, the supernatant had no suppressive activity, but on the other hand, the pellet had suppressive activity as strong as unseparated samples. From

Table 3. Suppressive effect of formalin-inactivated virus and HA samples on IgM PFC response in mice

Virus strain	IgM PFC/10^6 viable cells	Suppression %
none	720 ± 95	standard
A/CAM	12 ± 17	98%
A/WSN	57 ± 35	94%
A/MEL	12 ± 21	98%
A/PR/8	63 ± 19	91%
HA samples of A/PR/8	677 ± 51	N.S.

2048 HAU of formalin-inactivated virus was injected by i.p. route. SRBC was injected i.p. 2, days after infection.
N.S.; not significant, $P > 0.05$

this we conclude that viral particle itself had IS activity. To determine if HA molecules only could suppress the PFC response, we used A/PR/8 virus HA obtained as described above. 8192 HAU in 1 ml of HA sample had no IS activity (Table 3).

B cell clones producing IgM, IgA and IgG decreased after i.p. injection of influenza virus

We examined whether influenza virus would depress the number of B cell clones regardless of their specificity. Mice were injected i.p. with 4096 HAU of A/CAM virus and, 2 days later, the spleen cells were harvested and cultured in vitro, with 2 μg of PWM for 4 days. Protein A plaque assay was performed with cultured cells (Table 4). Less polyclonal IgM, IgA and IgG secreting cells were found among cultured spleen cells from infected mice than in those from normal mice. This demonstrated the decrease of B cell clones for

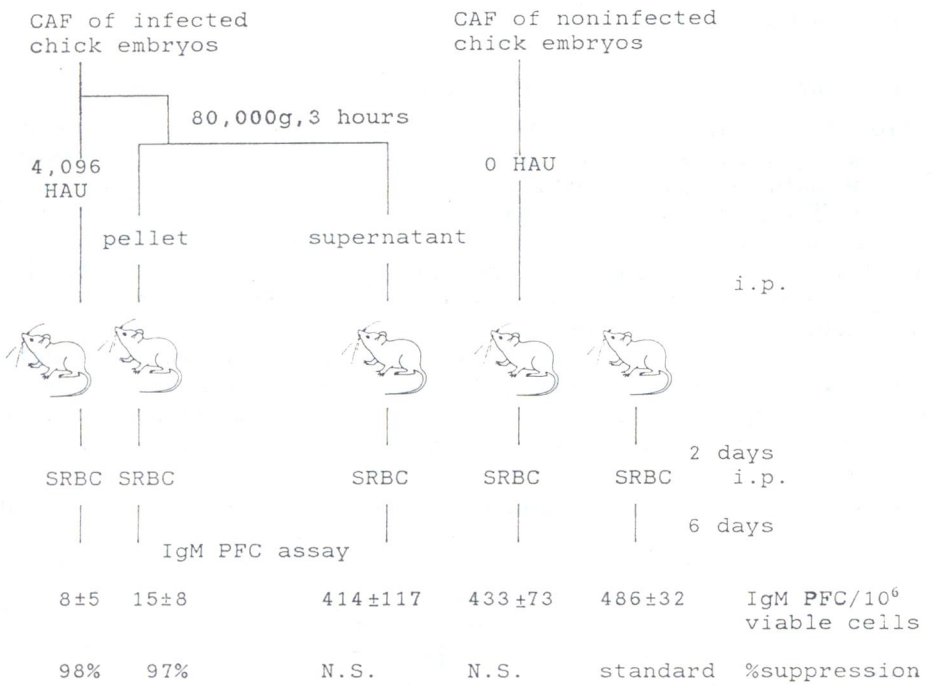

Fig. 1.
Suppressive effect of ultracentrifuged pellet of chorioallantoic fluid from infected chick embryos

CAF of chick embryos infected with influenza virus A/CAM was fractionated by ultracentrifugation at 80 000 g for 3 hr. Supernatant and pellet were separately injected i.p. to each of 4 to 5 mice.
CAF: chorioallantoic fluid
N.S.: not significant, $P > 0.05$

Table 4. Suppressive effect of influenza virus A/CAM on the number of polyclonal B cell clone in mice spleen

Mice source of cultured spleen cells	Polyclonal PFC/10^6 cultured cells IgM	IgA	IgG
Uninfected	16 240	720	2 008
Infected	2 720	192	384
Suppression (%)	83%	73%	81%

Mice were i.p. injected with virus A/CAM 4096 HAU, 2 days before preparation of spleen cells. Spleen cells from 2 mice were pooled and cultured 4 days with PWM 2 μg/ml. Harvested cells were assayed for polyclonal IgM, IgA and IgG PFC with protein A SRBC.
% suppression = 1 − (PFC of cultures of infected mice/PFC of cultures of normal mice) × 100%

IgM, IgA and IgG at the time of harvesting the spleen cells, i.e. 2 days after i.p. injection of the virus. We can assume from this fact that the suppression of PFC response by influenza virus might not be restricted for SRBC only.

The suppressive effect was T cell dependent

SRBC are a T cell dependent antigen. Therefore, we checked a T cell independent antigen, DNP-lys-Dextran-T 2000. As shown in Table 5, influenza virus A/CAM had no suppressive effect on the IgM PFC response to DNP. We believe that the IS effect caused by influenza virus might be a T cell dependent phenomenon and that B cells of the infected mice might not be affected.

To prove this assumption, we injected influenza virus to nude (nu/nu) mice, a congenitally athymic mutant, and assayed the IgM PFC response to SRBC. The nude mice injected i.p. with 4096 HAU of influenza virus A/CAM, responded to SRBC slightly higher than noninfected ones (Table 6). When we transferred the T cell enriched spleen population (2×10^8 cells) or thymus cells from normal thymic mice to nu/nu recipients and the virus was injected one week later, the suppression of PFC response was clearly demonstrated.

Table 5. Anti-DNP PFC response of mice infected with influenza virus

Days of infection before immunization with DNP	IgM PFC/10^6 viable cells	Suppression %
Non infected control	101 ± 15	standard
2 days	104 ± 37	N.S.
7 days	124 ± 24	N.S.

4096 HAU of A/CAM virus was injected i.p 2 or 7 days before immunization with DNP-lys-Dextran-T 2000 (1 mg protein). IgM PFC assay for DNP was performed 4 days after immunization, using SRBC coated with TNBS.
N.S.; not significant, $P > 0.05$

Table 6. Suppressive effect of influenza virus A/CAM on IgM PFC response to SRBC in nude mice

Virus, inoculated to nude mice	Donor mice of T cell	IgM PFC/10^6 viable cells of the recipient	Suppression %
Experiment 1			
A/CAM 4096 HAU	not transferred	53 ± 12	−65%
0 HAU	not transferred	32 ± 8	standard
Experiment 2			
A/CAM, 4096 HAU	uninfected	36 ± 11	69%
0 HAU	uninfected	117 ± 58	standard
Experiment 3			
0 HAU	infected with A/CAM 4096 HAU	230 ± 63	48%*
0 HAU	uninfected	444 ± 80	standard

Experiment 1: 4096 HAU of influenza virus A/CAM was injected i.p. to each of 4 nude mice, 2 days before immunization with 4×10^8 SRBC.

Experiment 2: 2×10^8 cells of T cell enriched population from normal Balb/c mice was transferred i.v. to each of 4 nude mice a week before infection. 4×10^8 SRBC was administered 2 days later.

Experiment 3: 2×10^8 cells of T cell enriched population from Balb/c mice infected i.v. 2 days before or noninfected Balb/c mice were transferred to nude mice. 4×10^8 SRBC were injected i.p. on the next day.

IgM PFC assay was performed 6 days after immunization in each experiment.

*: % suppression in the case of experiment 3 = 1 − (PFC of nude mice transferred with T cell from infected Balb/c mice/PFC of nude mice transferred with that from noninfected mice) $\times 100\%$.

The IgM PFC response in infected mice reached approximately 31% of non-infected ones (Table 6).

Depression of T cell activity supporting IgM PFC response in nude mice

Spleen T cells 2×10^8 from normal or infected Balb/c mice were transferred to nude mice. We compared the IgM PFC response of these recipients (Table 6). The T enriched population from normal mice supported PFC response stronger than that from infected mice.

Low CPA dose cannot relieve suppression

To clarify whether suppressor T cells are generated and interfere with the PFC response in infected animals, we injected a low dose of CPA, reported to affect precursors of T suppressor cells (20 mg/kg i.p.) to Balb/c mice infected with influenza virus A/CAM(4096 HAU)2 days before, on the same day or 2 days after the drug administration. The IgM PFC response to SRBC of these mice was measured. CPA could not relieve IS, which occurred in mice after i.p.

injection of influenza virus (data not shown). We assumed that suppressor T cell had no intimate correlation to the influenza virus caused IS.

Failure to transfer the suppressive activity

In order to determine whether any suppressive factor, humoral or cellular, was produced in mice upon i.p. virus injection, we transferred to normal recipients serum (0.4 ml) or spleen cells (2×10^8) from the mice 2 or 8 days previously infected with 4096 HAU of A/CAM virus. At the time of the

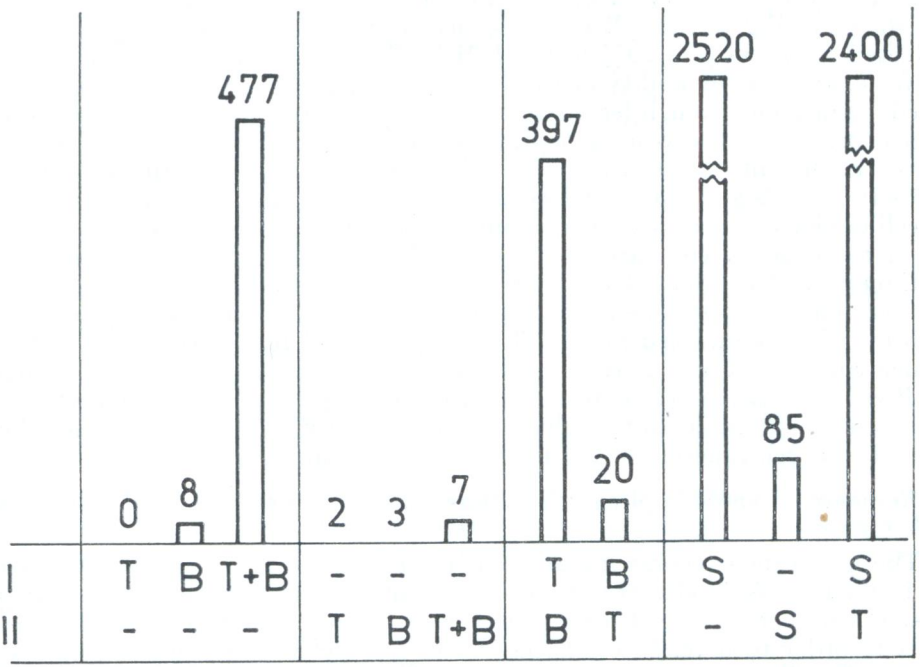

Fig. 2.

IgM PFC response in vitro to SRBC of spleen cells from normal and influenza virus infected mice, following fractionation and recombination

Mice were immunized with SRBC 4×10^7 7 days before and injected i.p. with influenza virus A/CAM 4096 HAU, 2 days before preparation of spleen cell suspension. Normal control mice were immunized only with SRBC. Spleen cell suspension from each 3 mice was separated to T (T cell enriched) and B (T cell depleted, cell population. 5×10^6 cells/ml were cultured in 96 well tissue culture dishes with SRBC, 2×10^8/ml. Recombined culture consisted of 2.5×10^6/ml from normal and 2.5×10^6/ml from infected mice, 5 days after establishment of culture, cells were harvested and IgM PFC assay was performed.
The last column in the right: This culture contained 3.5×10^6/ml of normal mice spleen cells and 1.5×10^6/ml of infected mice spleen T cells.
I — normal (control) mice; II — infected mice.
S = spleen cell suspension
Data indicate IgM PFC per 10^6 viable cells.

transfer the animals were given 2×10^7 SRBC. Neither serum nor spleen cells could alter the IgM PFC response of recipient mice (data not shown).

B cells from infected mice responted similarly as B cells from noninfected mice, when supplemented with T cells from un infected mice

To analyse the mechanism under which influenza virus depressed the immunological activity of mice, we assayed the in vitro PFC response of fractionated or unfractionated spleen cells from infected mice. 4096 HAU of A/ /CAM virus was injected i.p. 2 days before preparation of spleen cells. The spleen cells were separated to T cell enriched and T cell depleted fractions. As shown in Fig. 2, the IgM PFC response of unfractionated spleen cells from infected mice was 3 to 10% of the IgM PFC response from normal mice.

We examined the ability of the B cell (T cell depleted) and of T cell enriched population from infected mice to respond against SRBC. They were co-cultured with the complementary B or T cell populations from control mice. Each culture consisted of 5×10^6 cells/ml; mixed cultures included 2.5×10^6 B cells and 2.5×10^6 T cells. When cultured alone, T cell enriched or T cell depleted populations from control and infected mice gave only a marginal response as compared with that of the unfractionated spleen cells. Although the fractionated and thereafter co-cultured cells either from normal or infected mice responded not as good as the original cell populations, the culture which contained normal T cells responded higher than the culture which contained the T cells from infected animals, irrespective of the source of B cell population. These results clearly indicate that T cells from infected mice were responsible for the influenza virus induced PFC response depression and that B cell function of the infected mice remained intact.

Response of normal spleen cell culture was not suppressed by T cells from infected mice

The low responsiveness might occur either if T helper cells were deficient or if T suppressor cells were induced. In the next experiments we added a graded numbers of T cells of infected mice to normal unfractionated spleen cells in order to determine whether there was active suppression mediated by T cells. The cultures always contained constant numbers of cells, 5×10^6/ /ml. Even when the added T cells accounted for as much as 30% of whole cultured cell population, no suppressive effect was observed (Fig. 2). Thus, again the low responsiveness induced by influenza virus was either due to the deficiency of T helper cell population, or induction of suppressors other than T cells but not due to induction of T suppressor cells.

Discussion

Alterations in immune function have been observed to accompany many types of infection: especially viral infections were often studied concerning their immunomodulative activities. By these reports the actual forms of alteration vary depending upon kinds of hosts or viruses and upon other factors (Pelton *et al.*, 1980; Bendinelli *et al.*, 1982; Butler *et al.*, 1983; Wong *et al.*, 1984; Berencsi *et al.*, 1985). Enhancement or depression of immune

function may appear in the same host according to the stages of infection (Tingitella *et al.*, 1980). The mechanism of this phenomenon may be different. Direct effects on immunocompetent cells, lymphocytes, macrophages or polymorphonuclear leukocytes, and indirect effects through cellular or humoral factors such as interferon (Vignaux, F. *et al.*, 1980) may be involved. Influenza virus is sometimes a subject of experimental study concerning its IS activity on the host (Kleinerman *et al.*, 1976; Michael *et al.*, 1979; Abramson, 1982). It is well known that the course of influenza is often influenced by secondary bacterial infection. The IS effect of influenza virus in any way may facilitate secondary infection as a natural outcome (Abramson *et al.*, 1982; Gardner *et al.*, 1980, 1981). Not only the secondary bacterial infection, but also the modified pathogenesis by influenza virus itself must be considered when we study the result of immunomodulatory virus effect. This phenomenon may be important also with respect to application of some virus vaccines, since these may induce the IS state favouring current epidemics or activation of endogenous latent infection (Kantzler *et al.*, 1974; Arneborn *et al.*, 1980; Gallassi *et al.*, 1982).

The suppressive effect of influenza virus on the formation of antibody producing cells against the heterologous antigen, SRBC, for example, have been reported by some authors (Kavetsky *et al.*, 1977; Daniels *et al.*, 1981; Kiseleva *et al.*, 1982) but the mechanism of this phenomenon have not been fully elucidated. The present study was undertaken to examine the mechanism underlying the observed hyporesponsiveness of humoral antibody production to SRBC. Kavetsky *et al.* (1977) reported that intranasal infection of Balb/c mice with A/Hong/Kong/1/68 or A/PR/8 influenza viruses resulted in a significant inhibition of the formation of antibody producing cells in response to SRBC and the IS was not observed by heat-inactivated virus. In their report the IS continued for a long period (3 to 6 months). Kiseleva *et al.*, (1982) also reported that i.n. inoculation of A/Victoria/35/72 strain resulted in 96% suppression of PFC response to SRBC but the influenza virus strains having no pathogenicity or toxicity for mice did not lead to the development of IS.

In contrast to these reports, we could not observe an IS effect in Balb/c mice, infected i.n. with pathogenic or nonpathogenic strains of influenza virus. Intraperitoneal or intravenous inoculation of live or formalin-inactivated virus, pathogenic or nonpathogenic for mice, resulted in severe IS. For example, 4096 HAU of A/CAM strain inhibited PFC response against SRBC to less than 1% of the response of noninfected mice. Virus strains with no pathogenicity for mice also had suppressive effect as strong as pathogenic strains. The duration of immunosuppression did not exceed a month in our experiments with A/CAM strain.

It seems impossible to explain this discrepancy only by the differences among viruses or mice strains. In our experiments, i.n. infection induced spleen atrophy but no IS. Nizamov and Shuratov (1980) also reported spleen atrophy in i.n. infected mice, and at the same time they had shown similar IS as did we observe in the i.p. or i.v. infected mice only. Perhaps the mechanism of the latter phenomenon, which we observed in Balb/c mice inocul-

ated by i.p. route, may be entirely different from that described by other authors. Our conclusion is that the suppressive activity of i.p. injected virus influenza is not correlated to its pathogenicity or toxicity and that virus infection by respiratory route and nonrespiratory route may induce immunologically different situation in the host.

Berencsi *et al.* (1985) reported IS in mice by i.p. inoculation with adenovirus to i.p. injected SRBC only and demonstrated that peritoneal macrophages played an important role. In our case, i.v. injected virus also suppressed PFC response, thus it seems impossible to explain by only impairment of peritoneal macrophages, although the role of other macrophages cannot be denied. We confirmed that viral particles prepared by ultracentrifugation of chorioallantoic fluid of infected chick embryos had IS activity. Further we were interested which component of the virions was responsible. When HA was tested, the amount of 8192 HAU had no activity by i.p. injection. Therefore, we can assume that HA only was not sufficient for inducing IS activity, but we cannot conclude that HA molecules were not involved at all. It may be possible that HA molecules and other virion components cooperate in the IS activity. Other components besides HA molecules must be considered, but the HA molecule will be of particular interest concerning the immunomodulative effect.

Fontana and Weiner (1980) reported that HA of reovirus type 3 was responsible for generation of suppressor T cells in vitro. They demonstrated receptors on murine lymphocytes for reovirus type 3 HA, but not for the type 1 HA. As to influenza virus, Armstrong *et al.* (1981) reported that purified haemagglutinin from the H0N1, H1N1, H2N2, H3N2, and influenza B subtypes were mitogenic for normal spleen cells from Balb/c mice. Anders *et al.* (1984) demonstrated mitogenicity of UV-inactivated influenza virus type A of subtypes H1, H2, H3 and H6, and they reported that mitogenicity was inhibited by monoclonal antibodies directed against HA. The biological relevance of mitogenicity in vitro for the immunosuppressive activity in vivo is not clear at present, but it is correct to assume that direct interaction of virus with lymphocytes might be important also in vivo.

By our experiments in mice we can assume that the specificity for HA might not be so strict because viruses of different HA type (H1 for A/CAM, A/CAM, A/PR8, A/MEL and A/WSN; H3 for A/Hong Kong and also type B virus) exerted the IS activity of the same extent. If we would have used influenza virus of type H2, which was reported to have the strongest mitogenic activity (Anders *et al.*, 1984), we might have been able to examine the correlation between the intensity of IS in vivo and mitogenicity for lymphocytes in vitro.

The existence of murine lymphocyte receptors for influenza virus have not been clearly proved. We could not demonstrate viral antigen by FAT neither in lymph nodes nor in the spleen of i.p. injected mice. We cannot deny the direct action of virus on lymphocytes based on this result only. We must consider the functional alterations of numerous immunocompetent cells at various level during the course of viral infections. Daniels *et al.* (1981) reported about in vitro suppressive effect of influenzavirus on PFC response of

murine lymphocytes against SRBC. They also demonstrated the suppressive effect of also UV or heat-inactivated virus. This report indicates the importance of direct action or adsorption of virus on murine lymphocytes.

In our experiments, PFC response to DNP-lysine Dextran T2000, which is a T cell independent antigen, was not suppressed and we could not see the IS in athymic, nude mice. When we transferred normal T cells to these nude mice and tested the IS activity again, we proved these mice to be susceptible for IS activity of influenza virus.

Thus, we assumed that IS induced by influenza virus might be a T cell dependent phenomenon and B cell function might not be impaired. The T enriched population from normal mice more efficiently supported the PFC response in nude mice than that from infected donors when transferred to nude mice recipients. Then we attempted to determine whether induction of suppressor T cells was responsible for this weak activity of T cells from infected mice to support the IgM PFC response. We used low dose of cyclophosphamide, to which precursors of T suppressor cells are reported to be sensitive. Cyclophosphamide could not relieve IS. Moreover, in the transfer experiment to normal mice, active suppressor cells — T cells, macrophages or others — were not demonstrated among spleen cells from i.p. infected mice. In vitro PFC assay also proved no active suppression mediated by T cells. From these results we concluded that suppressor T cells were not important and that helper T cells might be functionally injured or reduced in number.

We assayed in vitro PFC response to estimate the function of T cells and B cells of infected mice. B cells of infected mice responded normally when they were cultured with normal T cells, but responded weakly when they were cultured with T cells from infected mice. This results also proved the normal function of B cells from infected mice. To estimate clearly the role of T helper cell deficiency, it would be useful to analyse by FACS (Fluorescence activated cell sorter) whether the number of T helper cells was reduced. According to our preliminary experiments no reduction of T helper cells was found.

In the case of IS by Coxsackie B3 virus in mice a deficit of macrophage accessory function and nonspecific suppressor T cells played an important role (Bendinelli *et al.*, 1982). Also in the case of IS by murine cytomegalovirus (Bixler and Boos, 1981), active suppressor cells, plastic adherent and resistant to anti-thymocyte serum, were responsible. We must also consider the role of macrophages, as suppressor cells or antigen presenting cells. But in our case, unfractionated spleen cells which contain macrophages, were not suppressive at in vivo transfer experiments. Thus, it seems proper to deny the strong suppressive activity of macrophages at least against the PFC response of mice infected with influenza virus.

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