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# COMPARISON OF THE ANTI-INFLUENZA ACTIVITIES OF ANTIBODIES AND IMMUNE SYSTEM CELLS ADMINISTERED INTRANASALLY TO SYNGENEIC MICE

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*Summary.* — A novel experimental approach was employed to investigate the protective role of cellular and humoral factors in antiviral immunity under conditions of their prophylactic administration into the tracheobronchial cavities of normal mice-recipients which were challenged 24 hr later with influenza virus A/PR/8/34 (H0N1). The highest protective effect was afforded by intranasal administration of immune serum (91.3%) as compared with splenocytes (65.2%). The addition of immune serum to cell suspensions or pooled lymphocyte and macrophage fractions previously isolated from the spleen increased the protective effect of these materials by 97–100% thus surpassing the protective effect of immune serum alone. The protective effect of passively administered cells from inflammatory exudates of peritoneal or tracheobronchial cavities was higher than that of a more concentrated splenocyte suspension; the results were comparable only if the number of splenocytes was 4- to 5-fold that of the exudate cells. The degree of the protective effect was strictly proportional to the concentration of immune system cells administered to the concentration of immune system cells administered to the syngeneic recipients.

*Key words*: *influenza virus*; *immune system cells*; *immune serum*; *antiviral immunity*

## *Introduction*

In vivo experiments on the protective role of various groups of immunocompetent lymphoid mouse cells have usually been carried out under conditions of intravenous adoptive transfer of the test cells of the immune system to syngeneic mice that had been previously X-irradiated to suppress their own lymphoid apparatus (Miller and Mitchell, 1968; Woodruff and Gesner, 1969; Rager-Zisman and Allison, 1976).

We are reporting the results of a novel experimental approach proposed by one of us (A.A.S.) aimed at comparative investigations on the role of the

protective effect of cellular and humoral factors of antiviral immunity. The method is based on a comparative study of anti-influenza activities of antibodies and immune system cells in the organisms of normal animals in the early stages of experimental influenza infection of various severity in inbred mice.

We found a marked protective effect not only of antibodies but also of different groups of immune system cells on the intensity of pathomorphological changes in the lungs and on the mortality of mice following experimental influenza infection. In addition to antibodies, a high protective effect was afforded by cells from inflammatory exudates of peritoneal and tracheobronchial cavities of immunized syngeneic mice.

## *Materials and Methods*

*Mice.* The experiments were carried out on adult (6-8 weeks old) CC57Br or C3H mice.

*Immunization of mice.* The animals were immunized by intraperitoneal (i. p.) administration of $10^6$ $EID_{50}$/0.2 ml of A/PR/8 influenza virus in the form of infected allantoic fluid. Control mice were immunized with Sendai and herpes simplex type 1 (HSV 1) viruses. Immunization of the donor mice did not result in the development of a respiratory infection, but stimulated the immunity against intranasal administration of a massive dose ($10^7-10^8$ $EID_{50}$) of homologous virulent virus after 14 or more days.

*Preparation of antibodies and immune system cells for administration to recipient mice.* The immunized mice were killed 7-8 days after immunization by total exsanguination from a. axillaris and subsequent perfusion of the animals with warm medium 199 through the left heart ventricle, to remove remnants of blood and antibodies from internal organs. Aseptically dissected lymphoid organs were homogenized in glass homogenizers in Eagle's medium at pH 7.2—7.4. The cell suspensions were repeatedly centrifuged at $200 \times g$ for 5—7 min and the pellet was treated with 0.83 mol/l $NH_4$ Cl to lyse the erythrocytes (Frederick and Bohl, 1976). After an additional two washings by centrifugation in Eagle's medium, the pellets were resuspended in medium 199 supplemented with 15% foetal calf serum that had been heated for 30 min at 56 °C.

*Obtaining of peritoneal exudate cells.* One day before dissection the immunized donor mice were additionally stimulated by i. p. administration of saline or homologous virus diluted 1 : $10^5$ with saline. After total exsanguination of the mice, 4-5 ml of medium 199 were injected into the peritoneal cavity. The cell-containing fluid was collected by a Pasteur pipette through the exposed peritoneum into siliconized bottles. The fluid was centrifuged twice and the cell concentration was adjusted as required by adding medium 199 containing calf serum.

*Obtaining of tracheobronchial washings.* After total exsanguination, a ligature was made on the trachea at the uvular region. A needle of a siliconized tuberculin syringe was introduced into the trachea below the ligature and 0.8 ml of medium 199 supplemented with 10% calf serum were slowly injected. Without removing the needle, the contents of the tracheobronchial cavity were sucked off slowly and stepwise. The cell suspension was washed in medium and adjusted to the required concentration (70—120 million cells per ml).

*Viability of cells* in the suspensions obtained, estimated by trypan blue staining, was 92—95%.

*Spleen cell suspensions* were prepared and their ability to adhere to the glass was determined according to Rager-Zisman and Allison (1976).

*Treatment of recipient mice.* Cell suspensions and blood sera from immunized and normal mice were administered to syngeneic animals intranasally (i. n.) under light ether anaesthesia (0.05 ml volumes) or in equal doses into the caudal vein (i. v.; 0.5 ml volumes). Twenty-four hr after transfer of the cell suspensions or sera, the recipient mice were challenged with three doses (30, 1 and 0.1 i. n. $LD_{50}$) of a mouse-adapted variant of influenza virus A/PR/8/34.

*Evaluation of morphological changes in mouse lungs.* The intensity of lesions was scrored as follows: 0 — no lung changes; + — 10—20% of the lung volume involved; ++ — 30—50% of the lung volume involved; +++ — 60—100% of the lung volume involved; ++++ — death of the mice. The mean morbidity indices and the protection rate were calculated per 20 mice (Anker *et al.*, 1978).

**Table 1. Protective effects of immune serum and splenocytes from immunized CC57Br mice given i.n. (I) or i. v. (II) to normal mice 24 hr before i. n. challenge with A/PR/8 influenza virus.**

| Mice given | | No. of mice with lung lesions of the indicated intensity | | | | Protection % |
|---|---|---|---|---|---|---|
| | | ++ | + | 0 | Total* | |
| Immune serum (titre 320) | I | 0 | 2 | 18 | 2.0 | 91.3 |
| | II | 9 | 9 | 2 | 27.0 | 0 |
| Immune splenocytes, $7 \times 10^6$ cells per mouse | I | 0 | 8 | 12 | 8.0 | 65.8 |
| | II | 5 | 11 | 4 | 23.0 | 0 |
| Immune splenocytes' ($0.7 \times 10^6$ cells per mouse) | I | 4 | 12 | 4 | 20.0 | 13.0 |
| | II | 5 | 15 | 0 | 25.0 | 0 |
| Normal splenocytes ($4 \times 10^6$ cells per mouse) | I | 5 | 20 | 5 | 23.2 | 0 |
| | II | 5 | 14 | 1 | 24.0 | 0 |
| Saline (control) | I | 6 | 11 | 3 | 23.0 | 0 |

* Total score per 20 mice.

*Titration of antibodies in blood sera and tracheobronchial washings.* The cellular elements in exudates were disintegrated in an MSE ultrasonic disintegrator (60 W, 20 kHz, 1.5 A, amplitude 7—8 μm, 20 sec). Cell debris was removed by centrifugation for 5 min at 200 × g. The supernatant fluids as well as the sera were heated for 30 min at 56°C and assayed in neutralization tests in chick embryos with 10-20 $EID_{50}$ of virus, occasionally also in haemagglutination inhibition (HI) tests with 2-4 haemagglutinating units of virus. Before examined in HI tests (Takátsy's micromethod), the test materials were absorbed with a 20% suspension of chick erythrocytes to remove spontaneous haemagglutinins. Removal of antibodies from lymphoid suspensions was checked in all experiments by determinig the virus neutralizing activities of cell suspensions in chick embryos.

*Interferon* in mouse sera was titrated by quantitative inhibition of haemadsorption caused by Sendai virus in L-cell cultures (Aksenov *et al.*, 1969).

*Virus in infected mouse lungs* was titrated by assaying 10% lung suspensions in chick embryos. The mean values were calculated from Student's tables.

*Testing of immune system cells for absence of virus neutralizing antibodies.* Normal mice were passively immunized by intravenous administration of highly active serum (titre 2560), which after 6 hr resulted in an antibody titre in the blood from 40 to 80. This corresponded to the antibody level in donor mice 7-8 days after immunization. Spleen suspensions from such animals were assayed as follows: without washing off the antibodies and after their thorough removal by perfusion of the immune animals through the heart with 50 ml of warm medium 199, or by low speed centrifugation of the cell suspension incubated for 30 min at 37° C.

*Anti-Θ serum* was prepared by immunizing AKR mice 5 times i. p. with thymocytes from CBA mice. The cell suspensions tested were freed of T lymphocytes according to Rager-Zisman and Allison (1976).

## *Results*

### *Comparative evaluation of the efficiency of passive immunization of animals by antibodies and immunocompetent cells*

In the first series of experiments we compared the efficiencies of i. n. and i. v. administration of (*1*) immune serum from mice immunized with A/PR/8/ /34 virus, with a titre of 320; and (*2*) splenocytes from immune and normal mice in doses of 7 or 0.7 million cells per mouse. I. n. administration of immune serum was the most effective. It protected 91.3% of the animals from lung lesions. Somewhat lower protection rate (65.2%) was obtained on i. n. ad-

**Table 2. Protective effect of splenocytes from passively immunized normal mice against experimental influenza infection in syngeneic animals**

| Materials administered 24 hr before challenge with virus | No. of mice with lung lesions of the indicated intensity | | | | | | Protection % |
|---|---|---|---|---|---|---|---|
| | ++++ | +++ | ++ | + | 0 | Total* | |
| Immune serum (titre 320) | 0 | 0 | 0 | 6 | 14 | 6 | 91.2 |
| Immune splenocytes after washing off ant body, $10^7$ cells per mouse | 4 | 1 | 3 | 6 | 6 | 31 | 53.7 |
| Normal splenocytes loaded with antibody, $10^7$ cells per mouse | 4 | 2 | 7 | 5 | 2 | 41 | 38.8 |
| Normal splenocytes loaded with antibody wh ch was subsequently washed, off, $10^7$ cells per mouse** | 12 | 3 | 2 | 2 | 0 | 61 | 9.0 |
| Immune splenocytes and macrophages, first separated and then pooled, unwashed, $10^7$ cells per mouse | 0 | 0 | 4 | 12 | 4 | 20 | 70.0 |
| Pool of immune spleen lymphocytes and macrophages + immune serum, $5 \times 10^6$ cells per mouse | 0 | 0 | 0 | 2 | 18 | 2 | 97.0 |
| Immune splenocytes + immune serum | 0 | 0 | 0 | 0 | 20 | 0 | 100.0 |
| Saline | 12 | 3 | 5 | 0 | 0 | 67 | 0.0 |

* Total score per 20 mice.
** This group consisted of 19 mice, all other groups of 20 mice.

ministration of $7 \times 10^6$ splenocytes freed of antibodies. The total intensities of lung lesions per 20 mice were 2 and 8 in the former and latter groups, respectively. Administration of a 10-fold lower dose of splenocytes resulted in a drop of the protection rate to 13%, which approached the control values (Table 1).

Intravenous administration of immune serum or splenocyte suspensions had no protective effect: the protection indices were the same as controls.

*The effect of antibodies on the efficiency of splenocytes from immunized donors*

The use of passive i. v. immunization of normal mice with highly active immune serum, with subsequent perfusion to free the animals of antibodies, made it possible to elucidate the probable role of humoral antibodies persisting in splenocyte suspensions in the protective effect of immune splenocytes (Table 2).

Marked protection of infected animals by administration of unwashed splenocytes from passively immunized mice was confirmed under conditions of infection of various severity. Normal splenocytes loaded in vivo with antibody also lowered the intensity of the pathological process. But after intensive washing of normal splenocytes from antibodies the protective effect of these cells disappeared — the indices of lung involvement in experimental and control animals were similar (61 and 67, respectively).

**Table 3. Protective effect of immune serum and immunocompetent cells against experimental influenza infection in syngeneic mice**

| Mice given | No. of mice with lung lesions of the indicated intensity | | | | | Protection | Virus titre in lungs |
|---|---|---|---|---|---|---|---|
| | +++ | ++ | + | 0 | Total* | % | (log $EID_{50}$) |
| Homologous immune serum | 0 | 0 | 16 | 4 | 16 | 68 | 1.75 |
| Normal serum | 4 | 14 | 2 | 0 | 42 | 16 | 2.00 |
| Homologous immune splenocytes | 0 | 4 | 16 | 0 | 24 | 52 | 1.30 |
| Normal splenocytes | 10 | 8 | 2 | 0 | 48 | 4 | Not done |
| Homologous spleen macrophages | 0 | 10 | 10 | 0 | 30 | 40 | 2.00 |
| lymphocytes | 1 | 6 | 13 | 0 | 26 | 48 | 2.50 |
| Heterologous immune splenocytes** | 9 | 8 | 3 | 0 | 46 | 8 | 3.25 |
| Medium 199 | 10 | 10 | 0 | 0 | 50 | 0 | 3.25 |

* Total score per 20 mice.
** Mice immunized with Sendai (parainfluenza 1) virus.
The cells were administered in doses of $6 \times 10^6$ to normal syngeneic CC57Br mice 24 hr before challenge with A/PR/8 virus. The mice were killed 7 days after challenge.

Lymphocytes and macrophages, isolated from a pool of spleen cells, were pooled and administered in a dose of $10^7$ cells per mouse. Without washing from antibodies they protected 70% of the animals, but after perfusion only 53.7%. The addition of immune serum to either unfractionated splenocyte suspensions or pools of isolated lymphocytes and macrophages markedly increased (by 97—100%) their protective effect, which was higher than that of the immune serum alone.

To check the specificity of the protective effect of antibodies and cell suspensions from mice immunized with influenza virus A/PR/8/34, mice were treated i. n. with similar materials obtained from mice immunized with the heterologous Sendai virus. The results presented in Table 3 showed that the latter had no effect on the course of the pathological process, as distinct from the regularly occurring action of immune serum and spleen cells from homologous immune donors. The course of influenza infection in recipient mice given splenocytes from immunized donors was less severe and more interferon accumulated in their sera as compared with unprotected animals or animals given immune serum. The levels of interferon in such mice were 4- — 6-fold higher than in the controls.

*Protective activities of lymphocytes and macrophages from inflammatory exudates*

Under conditions of passive prophylactic administration into the lower respiratory tract of normal mice of various humoral and cellular materials, serum antibodies afforded a more marked protection than lymphocytes and macrophages administered separately or as a pool. Out of the immunocom-

**Table 4. Protective effect of immune serum and spleen and tracheobronchial exudate cells against experimental influenza infection in syngeneic mice**

| Mice given | No. of mice with lung lesions of the indicated intensity | | | | | | Protection % |
|---|---|---|---|---|---|---|---|
| | ++++ | +++ | ++ | + | 0 | Total | |
| Immune serum (titre 320) | 0 | 0 | 0 | 10 | 20 | 6.6 | 90.6 |
| Immune splenocytes, $8 \times 10^6$ cells per mouse | 0 | 0 | 5 | 8 | 9 | 16.3 | 76.7 |
| Immune splenocytes treated with anti-Θ serum, $8 \times 10^6$ cells per mouse | 3 | 2 | 4 | 5 | 10 | 26.0 | 62.9 |
| Immune splenocytes, $2 \times 10^6$ cells per mouse | 2 | 1 | 2 | 3 | 12 | 18.0 | 74.3 |
| Immune tracheobronchial exudate cells, $2 \times 10^6$ cells per mouse | 5 | 2 | 5 | 3 | 5 | 39.0 | 44.3 |
| Immune tracheobronchial exudate cells treated with anti-Θ serum, $2 \times 10^6$ cells per mouse | 0 | 0 | 1 | 6 | 13 | 8.0 | 88.6 |
| Medium 199 | 15 | 6 | 2 | 1 | 1 | 70.0 | 0 |

* Total score per 20 mice.

petent cells the most active were cells of inflammatory exudates accumulating in the tracheobronchial or peritoneal cavities of immune mice under the action of small doses of homologous virus (10 $EID_{50}$) administered 24 hr before harvest of the cell suspensions. This conclusion was reached by comparing the prophylactic efficiencies of lymphocyte-macrophage cell populations obtained from the spleens or inflammatory exudates of peritoneal or tracheobronchial cavities of the same animals. We found that i. p. challenge of the immune mice resulted in complete inactivation of the A/PR/8 challenge virus within 18-24 hr. This made possible a safe i. n. administration to normal syngeneic mice of exudates obtained 24 hr after challenge, when reproduction of the challenge virus in the respiratory tract of experimental animals was excluded. Influenza virus repeatedly administered into the tracheobronchial cavities of immune animals was definitely inactivated 3-6 hr after inoculation.

As shown in Table 4, the protective effect of passively administered monocytes from inflammatory infiltrates of tracheobronchial cavity substantially surpassed that of a more concentrated suspension of cells from spleens of immunized mice. Comparable protection of mice by tracheobronchial exudate cells (74.3%) and splenocytes (76.7%) was observed only when the amount of the latter cells was 4-fold that of the former. Treatment of the tracheobronchial exudate cells with anti-θ serum in the presence of complement (Rager-Zisman and Allison, 1976) resulted in a 30% decrease of their activity (from 74.3 to 44.3%). Similar treatment of splenocytes caused a lower drop

**Table 5. Protective effect of immune serum and peritoneal and tracheobronchial exudate cells against experiental influenza infection in syngeneic mice**

| Mice given | No. of mice with lung lesions of the indicated intensity | | | | | Protection % |
|---|---|---|---|---|---|---|
| | +++ | ++ | + | 0 | Total | |
| Immune serum (titre 160) | 0 | 0 | 4 | 16 | 4.0 | 90.9 |
| Immune splenocytes, $10^7$ cells per mouse | 0 | 0 | 14 | 4 | 15.4 | 65.0 |
| Immune peritoneal exudate cells $10^7$ cells per mouse | 0 | 0 | 2 | 18 | 2.0 | 95.5 |
| $6 \times 10^6$ cells per mouse | 0 | 0 | 12 | 8 | 12.0 | 72.7 |
| Immune splenocytes, $2 \times 10^6$ cells per mouse | 1 | 3 | 5 | 1 | 28.0 | 36.2 |
| Immune tracheobronchial exudate cells, $2 \times 10^6$ cells per mouse | 0 | 2 | 8 | 4 | 17.0 | 60.0 |
| Medium 199 | 8 | 6 | 4 | 0 | 44.0 | 0 |

* Total score per 20 mice.

in activity (by 14.0%). This difference suggests that the lung exudate contained more T lymphocytes than the spleen cell suspension.

The protective activities of lymphocytes and macrophages of the inflammatory exudate from peritoneal and tracheobronchial cavities were comparable with the high efficiency of serum antibodies from the same animals. The marked protection was due to the concentration of the cell suspensions which probably resulted in additional production of type 1 interferon in the focus of influenza infection. Even peritoneal exudate cells from non-immunized mice lowered the course of infection in 22.7% of the animals. Two million splenocytes per mouse protected the mice-recipients 2-fold less than 10 million splenocytes (36.2% as compared to 65.0%) (Table 5).

## *Discussion*

We evaluated the protective effect of various materials from immune animals on a model represented by syngeneic normal mice. Twenty-four hr after i. n. administration to such animals of antibodies or various cell types from immunized or normal syngeneic mice, influenza infection of the respiratory tract was experimentally induced in the recipients. Under such conditions it was possible to evaluate the role paid in the prevention of pathological reactions of one or another protective material from syngeneic immune mice, the materials having been administered i. n. before inoculation of the animals.

The inefficiency of passive i. v. administration of antibodies or cell components from an immune organism in affecting the course of experimental influenza infection has been explained by difficulties in the transport of antibodies and even more so of lymphocytes and macrophages on to the surface of the respiratory tract mucosa, the main place of interaction of virus

with susceptible epithelial cells. The barrier of lung capillaries proved to be little permeable to antibodies and immune system cells that had been administered i. v. Intranasal administration of antibodies or immune splenocytes offers more favourable conditions for a protective effect. Penetrating into the trachea and bronchi of anaesthetized mice, these materials come into direct contact with the developing infectious process and thus show an optimal protective effect.

We found a much stronger prophylactic effect of passively administered antibodies as compared with lymphocyte or macrophage suspensions applied each separately or with their natural pools in the form of splenocytes or lymphocytes from the blood or lymph nodes of immune animals.

The rather high anti-influenza activity of inflammatory exudate cells could probably be explained by the fact that inflammatory exudates do not consist of just any cells of the immune system, but that they selectively contain mainly specific clones consistig of blast-transformed immunocompetent T lymphocytes and immune macrophages. Data about the role of cytotoxic T cells in the release of virus from infected tissues are scarce. Informations concerning cell-mediated immunity have been based on the activities of circulating or spleen cells (Rabinowitz and Lipton, 1974; Shultz *et al.*, 1974; Rustigian *et al.*, 1975). The prevalence of T lymphocytes in infected foci as compared with peripheral blood and lymphatic organs has been reported (Raff and Owen, 1971; Hapel and Gardner, 1974; Morishima and Hayashi, 1978).

In studying the protective role of cellular factors in anti-influenza immunity we paid especial attention to the evaluation of cell populations concentrated in artificially induced inflammatory exudates in peritoneal and tracheobronchial cavities of immune animals. The protective effect of such cells administered i. n. 24 hr before inoculation of the recipients with homologous influenza virus was significantly more marked than the protective effect of similar cells obtained under the same conditions from other anatomic parts of the animals. By their high protective effect the inflammatory exudate cells approached the activity of virus neutralizing antibodies. The probable cause of the high protective efficiency of this group of cells could be their specific influx and accumulation in various cavities of the immune animal, into which homologous virus is administered. Selective influx of immunocompetent lymphocytes and immune macrophages into foci of inflammatory reactions results in their quantitative dominance in comparison with the reserve supplies of the same cells in the spleen, lymph nodes and blood of immunized animals.

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