CELL-MEDIATED IMMUNITY IN EXPERIMENTAL PARAINFLUENZA TYPE 3 VIRUS INFECTION

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PLATES XXXIII-XXXV

PARAINFLUENZA viruses are mainly reported as causing respiratory-tract infections in children. Of the four types, parainfluenza type 4 is the least and type 3 is the most frequently reported. Parainfluenza type 3 (PI-3) is found more often in very young children. The lungs are the main site of infection and local immunity develops early (Smith *et al.*, 1966; Kasel *et al.*, 1969).

Reports on cell-mediated immune responses during experimental respiratory-virus infections are few (Waldman and Ganguly, 1974). Delayed hypersensitivity has been demonstrated after parenteral administration of inactivated respiratory syncytial virus (Forsyth, 1968; Tremonti and Jackson, 1969) and inactivated influenza virus (Feinstone, Beachey and Rytel, 1969). There are some conflicting reports of suppression of cell-mediated immunity (CMI) in respiratory viral infection (Kantzler *et al.*, 1974; Kauffman *et al.*, 1974). Even the reports on the development of delayed hypersensitivity after experimental inoculation of live PI-3 virus are contradictory. Tremonti and Jackson (1969) failed to induce a delayed hypersensitivity response by intranasal inoculation of live PI-3 virus whereas Wetherbee (1973) could induce it. We have produced PI-3 virus infection in the respiratory tract of guinea-pigs and have studied the development of systemic CMI for 70 days, using dermal reactivity, leucocyte-migration inhibition (LMI), macrophage-migration inhibition (MMI), macrophage aggregation (MA) and adoptive spleen-cell transfer as indicators.

MATERIALS AND METHODS

Media

Hanks's balanced salt solution (HBSS) containing penicillin 100 units/ml, streptomycin 100 µg/ml and heparin 5 units/ml and Eagle's minimum essential medium with Earle's basic salt solution (MEM) were prepared according to Lennette and Schmidt (1964). MEM also contained glutamine 3% (w/v), penicillin 100 units/ml and streptomycin 100 µg/ml. Enriched MEM was prepared by the addition of 10% (v/v) heat-inactivated bovine serum to MEM. The pH of the final medium was adjusted to 7.2 with 1.4% (w/v) sodium bicarbonate.

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Animals

Healthy guinea-pigs of both sexes weighing 300–400 g, bred and maintained in our institute animal house, were used. They were given a diet of Bengal gram and vegetables and water *ad lib*. They were kept in separate cages.

Virus

Parainfluenza 3 virus was used. Pools of infective virus were prepared in HeLa monolayer cell culture. Cells infected with the virus were harvested after incubation for 3 days. The infected tissue-culture fluid was centrifuged at 2000 r.p.m. for 10 min. and the supernatant fluid was stored at -60°C .

Titration of virus

From each of a series of tenfold dilutions of virus suspension in Eagles maintenance medium, 0.2 ml was inoculated into three sets of HeLa cell-culture tubes. After incubation for 3 days, the tubes were washed twice with 1 ml of MEM; 1 ml of 0.1% guinea-pig erythrocyte suspension in MEM was added to each tube and the tubes were kept at 4°C for 30 min. The cultures were examined microscopically for evidence of haemadsorption. The titre, calculated by the method of Reed and Muench (1938), was $10^{5.5}$ 50% tissue-culture infective doses (TCID₅₀)/ml.

Infection of animals

Virus suspension (0.2 ml) was instilled intranasally into 20 guinea-pigs. Groups of three animals were examined for development of CMI after different time intervals. Their sera were also tested for virus-neutralising antibody by haemadsorption inhibition.

Antisera

Undiluted virus suspension (0.2 ml of $10^{5.5}$ TCID₅₀/ml) was instilled intranasally into four guinea-pigs. The animals were kept in isolation and bled by cardiac puncture after 21 days. Sera were stored at -20°C . The pooled sera had a virus neutralising antibody titre of 1024.

Measurement of cell-mediated immunity

Antigen. Dilutions of virus containing 10^2 or 10^3 TCID₅₀/ml were used for eliciting dermal reactivity, LMI, MMI and MA. The antigen was inactivated by heating in a waterbath at 60°C for 1 h. To ensure that there was no live virus in the preparation, 0.2 ml was inoculated into HeLa cell monolayer culture tubes and tested for haemadsorption of guinea-pig erythrocytes after incubation at 37°C for 3 days. They did not show any haemadsorption.

Delayed skin hypersensitivity. From each of the two antigen preparations, (10^2 and 10^3 TCID₅₀/ml), 0.1 ml was injected intradermally into different spots in an area of shaved abdominal skin of test animals; 24 and 48 h later, both sites of inoculation were examined for induration and erythema.

Macrophage migration-inhibition test. The method of David *et al.* (1964) as described by Agarwal and Sundararaj (1976, 1977) was used. The peritoneal macrophages were collected from virus-infected guinea-pigs 48 h after the intraperitoneal injection of 15 ml of 3% (v/v) peptone water. They were harvested in 150–200 ml of cold HBSS containing heparin 5 units/ml. Any erythrocytes in the macrophage suspension were lysed by keeping the centrifuged macrophage cell pellet in 5 ml of 0.83% (w/v) ammonium chloride solution. Separate plastic chambers containing macrophages in microcapillaries were filled with enriched MEM (3 ml) and contained 0.1 ml of either 10^2 or 10^3 TCID₅₀ antigen/ml or no antigen. The area of macrophage migration

in control and test chambers was recorded with a camera lucida. An average of the migrations from five capillaries was taken and the percentage of migration inhibition was calculated from the formula:

$$\frac{\text{area of control migration} - \text{area of test migration}}{\text{area of control migration}} \times 100.$$

Leucocyte migration-inhibition test. This was done by the method of David *et al.* (1964). Samples of blood (10 ml) were collected in tubes containing 0.25 ml of heparin (250 units) and allowed to stand for 1 h for the erythrocytes to settle. The plasma was collected and centrifuged at 1000 r.p.m. for 10 min. To the cell pellet, 5 ml of 0.83% (w/v) NH_4Cl was added and the suspension was allowed to stand for 5 min. After the erythrocytes had lysed, the leucocytes were collected by centrifugation at 1500 r.p.m. for 2 min. The cells were then washed thrice with HBSS and suspended in enriched MEM containing antibiotics, and adjusted to a concentration of $2-3 \times 10^6$ cells/ml. The viability of the cells was tested by trypan-blue exclusion. The rest of the procedure was the same as for the MMI test and the area of migration was calculated after incubation of the chambers at 37°C for 24 h.

Macrophage-aggregation test. This test was performed as described by Lolekha, Dray and Gotoff (1970). Two ml of enriched MEM containing 2×10^6 peritoneal-exudate cells (PEC) were cultured in siliconised conical tubes (15×150 mm) in the presence of 10^3 TCID₅₀ of virus antigen. Antigen was not added to the control tubes. All the tubes were incubated at 37°C in 5% CO_2 for 24 h. After incubation the cells were suspended by gentle mixing. One drop of cell suspension was placed on a slide and observed by light microscopy ($\times 100$). The test was done in duplicate and the results were graded from + to + + + + according to Lolekha *et al.* (1970) by counting the number of cells forming a clump (+ = 5-9 cells, + + = 10-15, + + + = 16-25, + + + + = clumping of many cells which settled below the supernatant fluid).

Spleen-cell transfer

This was done as described by Sundararaj and Agarwal (1977). Guinea-pigs experimentally infected with virus were killed 60 and 70 days after infection and CMI was measured. The spleens, removed and rinsed thrice with MEM containing penicillin 100 units/ml and streptomycin 100 $\mu\text{g}/\text{ml}$, were snipped into small pieces. These pieces were forced through a 40-mesh stainless-steel sieve. The spleen cells were suspended in MEM and their viability was checked by trypan-blue exclusion. More than 95% of the cells were viable. The cells were counted in a Neubauer chamber and the suspension was adjusted to 10^8 - 10^9 cells/ml. The suspension (0.5 ml) was injected intracardially into two normal guinea-pigs which were killed on the 3rd day. LMI, MMI and MA were measured. A piece of spleen removed from an infected guinea-pig was ground well, suspended and centrifuged. The supernate was inoculated into HeLa cell monolayer cultures. After 4 days, viable virus in the cells was sought by haemadsorption and none was found.

Virus-neutralising antibody in serum

Sera were held at 56°C for 30 min. Twofold dilutions of the test serum were prepared in MEM. An equal volume (0.1 ml) of virus was added to each serum dilution and the serum-virus mixtures were incubated at room temperature for 1 h.

After incubation, 0.2 ml of each serum-virus mixture was inoculated into three sets of HeLa cell cultures. After incubation at 35°C for 3 days the test was read, haemadsorption inhibition indicating the end point as follows. The cultures were washed twice with MEM then 1 ml of a 0.1% (v/v) suspension of guinea-pig erythrocytes was added to each tube. After incubation at 4°C for 30 min., the tubes were examined microscopically for haemadsorption. Complete inhibition of haemadsorption was taken to indicate neutralisation. The serum end point was estimated by the method of Reed and Muench (1938).

Histological examination of tissue from both lungs and from the trachea was made on all

experimentally infected guinea-pigs, which were killed after different time intervals. The tissues were fixed in 10% formol saline. Sections were cut at 4 μ m and stained with haematoxylin and eosin.

RESULTS

The concentrations of virus antigen that did not, by themselves, increase the LMI or MMI when added to migration chambers containing macrophages or leucocytes from non-immune guinea-pigs were determined. These antigen concentrations of virus suspension could safely be used for inducing CMI in macrophages or leucocytes from PI-3-infected guinea-pigs. The results obtained in virus-infected guinea-pigs after various time intervals and by different indicators of CMI were as follows.

Dermal reactivity

At no time during the course of 70 days of experimental virus infection could dermal reactivity be elicited by intradermal injection of 0.2 ml of $10^{2.0}$ or $10^{3.0}$ TCID₅₀ antigen/ml. Erythema or induration could not be found 24–48 h after injection.

Macrophage-migration inhibition (table 1)

In the first group, 10 days after infection, one guinea-pig (no. 5) showed increase in MMI at both the concentrations of antigen. Twenty days after infection, all the three guinea-pigs (nos. 6, 9 and 10) showed good inhibition after the injection of the 10^3 TCID₅₀ preparation. But after infection with the 10^2 TCID₅₀ preparation, only two guinea-pigs (nos. 6 and 10) showed increased MMI. Thirty days after infection, all the three guinea-pigs (nos. 3, 11 and 12) showed increased MMI at both concentrations of antigen. Similar results were obtained in guinea-pigs examined 40, 50, 60 and 70 days after infection.

Leucocyte migration inhibition (table I)

In the first group of three guinea-pigs, some inhibition was induced in the leucocytes from two (nos. 7 and 8) 10 days after infection, by the addition of the 10^3 TCID₅₀ antigen preparation to the migration-chamber fluid. However, with the 10^2 TCID₅₀ antigen preparation in the migration-chamber fluid, leucocytes from none of the guinea-pigs showed inhibition. Twenty days after infection, the results were generally similar except that the increase in LMI was more pronounced and the leucocytes from guinea-pig no. 10 showed increased LMI with the 10^2 TCID₅₀ antigen preparation. Thirty days after infection, the leucocytes from all the guinea-pigs (nos. 3, 11 and 12) showed considerable increase in LMI with the 10^3 TCID₅₀ preparation in the migration-chamber fluid. After addition of the 10^2 TCID₅₀ preparation, however, two guinea-pigs (nos. 3 and 12) showed increased LMI. Forty days after infection, all the

three guinea-pigs (nos. 4, 13 and 14) showed increased LMI after addition of antigen at both concentrations. At 50, 60 and 70 days after infection, increased LMI was induced by both these concentrations of the antigen in all the guinea-pigs studied (nos. 15, 17 and 18).

Macrophage aggregation (table I)

Macrophages were seen to aggregate in the presence of antigen preparation 10^3 TCID₅₀/ml in all the guinea-pigs 20 days and more after infection. In control animals, there was no aggregation of macrophages.

Virus-neutralising antibody (table I)

The virus-neutralising antibody was present 10 days after infection (titre of 32, 64 and 64 in guinea-pigs nos. 5, 7 and 8, respectively). Titres increased to 512 and 1024 and remained at this high level even up to 60 and 70 days after infection.

TABLE I

Development of cell-mediated immunity in guinea-pigs after experimental infection with parainfluenza 3 virus

Guinea-pig no.	Days after infection	MMI (percentage)* by antigen preparation		LMI (percentage)* by antigen preparation		MA by antigen 10^3 TCID ₅₀	Titre of virus-neutralising antibody
		10^3 TCID ₅₀ /ml	10^2 TCID ₅₀ /ml	10^3 TCID ₅₀ /10 ml	10^2 TCID ₅₀ /50ml		
5	10	30.1	28.9	6.6	3.6	Not done	32
7	10	24.5	24.1	42.2	22.3	+	64
8	10	12.0	1.6	52.0	26.3	+	64
6	20	37.6	31.6	16.4	12.0	Not done	128
9	20	56.2	12.5	36.1	16.6	++	128
10	20	75.2	44.2	65.9	51.6	++	512
3	30	77.3	70.0	81.6	57.5	Not done	512
11	30	52.7	44.3	31.3	2.5	+++	512
12	30	65.7	46.3	57.8	47.7	+++	1024
4	40	64.7	40.9	33.2	41.3	+++	1024
13	40	37.7	36.7	50.7	28.8	+++	1024
14	40	40.5	20.6	54.2	32.9	+++	512
15	50	45.2	35.1	40.3	40.1	+++	1024
16	Died	...	...	...	...	...	...
17	60	51.3	46.2	50.2	44.1	+++	1024
18	70	40.5	37.3	56.3	50.7	++	512
1	Uninfected control	3.8	2.1	3.1	1.9	—	8
2	Uninfected control	5.4	3.2	5.3	2.8	—	8

TCID₅₀ = 50% tissue-culture infective dose.

MMI = macrophage-migration inhibition; LMI = leucocyte-migration inhibition; MA = macrophage aggregation.

+ = Aggregation of 5-9 cells; ++ = aggregation of 10-15 cells; +++ = aggregation of 16-25 cells; — = no aggregation.

* See Materials and methods for method of calculation.

Adoptive transfer of cell-mediated immunity (table II)

Spleen-cell transfers were made from two guinea-pigs (nos. 17 and 18) 60 and 70 days after infection into each of two normal seronegative guinea-pigs. All the immune recipient guinea-pigs into which the adoptive transfers were made showed good inhibition in the LMI and MMI tests. Dermal reactivity was, however, not present in these recipient animals when they were tested by intradermal injection of both the dilutions of antigen.

TABLE II

Adoptive transfer of cell-mediated immunity to guinea-pigs by immune spleen cells of guinea-pigs infected with parainfluenza 3 virus

Guinea-pig no.	Days after infection	MMI (percentage)* by antigen preparation		LMI (percentage)* by antigen preparation	
		10 ³ TCID ₅₀ /ml	10 ² TCID ₅₀ /ml	10 ³ TCID ₅₀ /ml	10 ² TCID ₅₀ /ml
17	60	51.3	46.2	50.2	44.1
17/1	60	63.3	46.4	64.1	42.2
17/2	60	62.6	50.0	59.3	47.2
18	70	40.5	37.3	56.3	30.7
18/1	70	53.1	46.9	61.6	55.0
18/2	70	43.4	41.4	78.1	58.4

Footnotes as in table I.

Histopathology

Ten days after infection, the lungs showed severe congestion and some areas of interstitial pneumonitis. The alveolar capillaries were dilated and ruptured in places. There were areas in which the alveolar spaces were completely obliterated and the septa were thickened, hypercellular and infiltrated with chronic inflammatory cells. In the trachea, the capillaries of the submucosa were dilated and there was a diffuse infiltration of mononuclear cells. Twenty days after infection the septal thickening and obliteration of the alveolar spaces in the lungs had increased. The capillaries were dilated and ruptured in many places (figs. 1 and 2). The histopathological appearance of the trachea at this time was similar to that at 10 days after infection, except that the inflammatory mononuclear-cell response was more severe (fig. 3). Thirty days after infection, the histopathological changes in the lungs and trachea had progressed further. Forty days after infection the lungs showed marked congestion of the alveolar septa, dilatation and rupture of capillaries and a heavy mononuclear cellular response. Focal areas of intraalveolar haemorrhages were also present. The trachea showed a diffuse infiltration with chronic inflammatory cells and dilatation of capillaries (figs. 4 and 5). The general appearances were those of interstitial pneumonitis. Similar histopathological features were found in the lungs and trachea of guinea-pigs 50 days after infection. They were less marked 60 and 70 days after infection.

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FIG. 1. —Lung, 20 days after infection. Shows hypercellularity and heavy mononuclear-cell infiltration of alveolar septa and obliteration of air spaces. Haematoxylin-eosin (HE). $\times 112$.

FIG. 2.—Lung, 20 days after infection. Note the marked obliteration of alveolar spaces and mononuclear-cell infiltration. HE. $\times 450$.

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FIG. 3.—Trachea, 20 days after infection. Note the focal aggregate of chronic inflammatory cells. HE. $\times 450$.

FIG. 4.—Lung, 40 days after infection. Shows marked dilatation of capillaries, rupture and haemorrhages and a diffuse infiltration of interalveolar septa with mononuclear cells. Note the obliteration of air spaces. HE. $\times 600$.

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FIG. 5.—Trachea, 40 days after infection. Shows a diffuse infiltration with chronic inflammatory cells and dilatation of blood capillaries in the submucosa. HE. $\times 600$.

DISCUSSION

Our results showed that experimental respiratory infection with PI-3 virus in the guinea-pig produced a systemic cell-mediated immunity which persisted for at least 70 days after infection. It is interesting that dermal reactivity was not found at any time during the course of respiratory-tract infection even though cellular immunity was present, as indicated by LMI, MMI, MA and adoptive cell-transfer experiments. Such a dissociation of dermal reactivity from other manifestations of CMI has been reported by us (Sundararaj and Agarwal, 1977) and others (Osebold, Pearson and Medin, 1974).

The development of CMI, as shown by an increase in migration-inhibition factors, had begun by the 10th day after infection although, by then, evidence of CMI was found in only one guinea-pig, and in that it was elicited only by antigen at a concentration of $10^{3.0}$ TCID₅₀/ml.

The local infection was established in the lung tissue by the 10th day as shown by the presence of areas of interstitial pneumonitis. Good CMI appeared 20 days after intranasal instillation of the virus and persisted for at least 70 days. This was shown by the increase in the LMI and MMI in animals killed from the 30th to the 70th day after infection. The CMI could be adoptively transferred to non-immune guinea-pigs by immune spleen-cell transfer even in the later stages of experimental infection, i.e., 60 and 70 days after infection. This was amply shown by the increased LMI and MMI in the recipient guinea-pigs. The recipient animals, like the donors, did not show any dermal reactivity.

The development of CMI could be correlated with the presence of histopathological changes in lung and tracheal tissue. Ten days after infection, when the virus had produced focal areas of interstitial pneumonitis, the CMI had just started appearing. The gross pathological lesions were seen up to 70 days after infection. Forty days after infection the lung tissue showed a slight clearing; the alveolar septa were less thickened and some alveolar spaces could be made out. However, there was marked dilatation and rupture of capillaries. Considerable interstitial pneumonitis was present up to the 50th day and it was less marked by the 70th day after infection. The inflammatory cell response in the lower part of the trachea had diminished by the 40th day. During this period the systemic CMI was certainly increased and was counteracting the local respiratory-tract infection. We feel that systemic cellular immunity might have an important role in addition to the local immunity in determining the course of the parainfluenza type 3 respiratory-tract infection. Humoral antibodies also appeared and progressively increased during the course of infection; these may be supplementing cell-mediated immunity in the localisation and clearing of viral pneumonitis.

SUMMARY

Local respiratory-tract infection was produced experimentally in guinea-pigs by intranasal instillation of a suspension of parainfluenza virus type 3.

Histologically, interstitial pneumonitis developed within 10 days and persisted for at least 70 days.

Cell-mediated immunity was measured at intervals for 70 days after infection. Dermal reactivity could not be elicited. Leucocyte-migration inhibition and macrophage-migration inhibition were increased. Macrophage aggregation was present. Increased cell-mediated immunity could be transferred from infected donor animals to normal recipient animals by adoptive spleen-cell transfer even 60 and 70 days after infection.

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