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Suppressor cells induced by influenza virus inhibit interleukin-2 production in mice

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SUMMARY

PR8 virus depressed interleukin-2 (IL-2) and natural killer (NK) cell activity in BALB/c infected mice. IL-2 production was not dependent on (i) a decreased number of T cells or (ii) a primary defect in IL-1 production, but on a T-suppressor cell subpopulation. In fact, when T suppressor cells were removed from infected spleen cells, we observed normal levels of IL-2 activity.

INTRODUCTION

The influenza virus is a myxovirus, and provides a continuous threat of epidemic disease in man. Detailed information is now available on the viral genome (role of gene reassortment and mutation in the generation of antigenic shift and drift) and on complete amino acid sequences of two surface glycoproteins (Both *et al.*, 1983; Colman, Varghese & Laver, 1983; Gerhardt *et al.*, 1981; Lamb & Choppin, 1983; Laver & Valentine, 1969). At the same time, it has been known that neutralizing antibodies and cytotoxic T lymphocytes (CTL) play an important role in the clearing of influenza virus and in the recovery phase of infection (Ada & Jones, 1986; Ennis, 1982). In fact there is evidence for the protective role of the neutralizing anti-HA antibody (Burlington *et al.*, 1983), which prevents the entry of the viruses into susceptible cells by blocking the virus association with cellular receptors. Moreover, the antibody may modify infection by the lytic action of complement or by antibody-dependent cell-mediated cytotoxicity (ADCC) (Hashimoto, Wright & Kerzon, 1983; Perrin *et al.*, 1976). Also, the influenza virus infection induces production of CTL cells that in turn protect the infected host by recognizing and killing virus-infected target cells (McMichael *et al.*, 1986; Taylor & Askonas, 1986; Wraith & Askonas, 1985).

In contrast, very few studies are analysing the changes of host immune response during influenza virus infection. Some investigators have found that the influenza virus infection can inhibit some cell-mediated immune functions (Karpovich *et al.*, 1983; Kantrler *et al.*, 1974; Reed, Olds & Kisch, 1972; Woodruff & Woodruff, 1976). Our previous studies demonstrated that mice infected *in vivo* with PR8 influenza virus showed a morphological alteration of thymocytes and thymic reticulo-epithelial cells (TREC), a serum thymic activity (STA) fall associated with both, and a decreased response to mitogen specific to T lymphocytes (Del Gobbo *et al.*, 1985).

The present study was carried out to analyse the impairment of cellular and natural immunological functions in PR8 influenza virus-infected mice and to identify the involved mechanism.

Our data indicate that PR8 influenza virus infection results both in depressed interleukin-2 (IL-2) production and decreased natural killer (NK) cell activity by stimulating T-suppressor cell subpopulations.

Abbreviations: ADCC, antibody-dependent cell-mediated; Con A, concanavalin A; CPE, cytopathic effect; CTL, cytotoxic T lymphocytes; CTLL, cytotoxic T-lymphocytes line; FCS, fetal calf serum; HA, haemagglutinin; IL-1, interleukin-1; IL-2, interleukin-2; NK, natural killer; PMA, phorbol myristic acetate; STA, serum thymic activity; Th, T helper; TREC, thymic reticulo-epithelial cells; Ts, T suppressor.

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MATERIALS AND METHODS

Mice

BALB/c mice, 4–5 weeks old, provided by Charles River (Calco, CO), were used in these studies.

Virus

A/PR/8/34 influenza virus (H_1N_1) was grown in the allantoic cavity of 10-day-old embryonated eggs; 48 hr later, the allantoic fluid was harvested. The virus was stored at $-70°$.

Virus inoculum

BALB/c mice were intranasally (i.n.) inoculated under light ether anaesthesia with 50 μl of PR8 virus suspension, containing 2×10^5 or 1×10^5 cytopathic effect (CPE) 50% units/ml, i.e. 1×10^4 and 5×10^3 CPE 50% units, respectively.

Preparation of spleen cell suspensions

At various time intervals, control and infected mice were killed by cervical dislocation and spleens were aseptically removed. The cell suspension was obtained from individual spleens by passage through a sterile syringe and by subsequent filtering

through a Nytex mesh. Cells were washed twice and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM/ml), 100 U/ml penicillin and 100 μg/ml streptomicin (Flow Laboratories, Irvine, Ayrshire, U.K.). The viability, as determined by trypan blue exclusion, was >95%.

Interleukin-2 assay

IL-2 production. Murine IL-2 production was induced by stimulating individual spleen cells (1×10^7/ml) from control and infected mice with 2 μg/ml Con A and 10 ng/ml phorbol myristic acetate (PMA) (Sigma, St Louis, MO). Con A and PMA concentrations were optimal for maximum IL-2 production (data not shown). After 48-hr incubation at 37°, the supernatant was collected and stored at −20° until use.

IL-2 titration. The assay was based upon the method of Gillis *et al.* (1978). Briefly, the supernatants (two-fold serially diluted in medium from 1:2 to 1:256) were added into 96-well microtitre plates (Flow Laboratories) containing an IL-2-dependent cytotoxic T-lymphocyte line (CTLL) cell suspension (1×10^5/ml). All assays were performed in triplicate and incubated for 24 hr at 37°. Control wells received both the complete culture medium and the mitogen-containing medium, instead of dilution samples. After 20 hr incubation, 1 μCi [^{3}H]thymidine (TdR, specific activity 86 μCi/mml; Amersham International, Amersham, Bucks, U.K.) was added and 4 hr later cells were harvested by a semi-automatic cell harvester. The radioactivity was measured as counts per minute (c.p.m.) in a β counter. IL-2 activity was measured as [^{3}H]TdR incorporation by the IL-2-dependent CTLL clone, and number of units was expressed as the reciprocal of supernatant dilution producing 50% maximal [^{3}H]TdR incorporation with reference to a standardized IL-2 preparation (Ronchese *et al.*, 1985). The number of units was calculated using the computer program described by Sette *et al.* (1986).

NK cytotoxicity assay

The NK cell activity was determined as by Herberman, Nunn & Larvin (1975). Briefly, YAC-1 cells (a Moloney virus-induced mouse T-cell lymphoma of A/SN origin), used as target cells, were labelled with sodium^{51}chromate (New England Nuclear, Boston, MA) for 1 hr at 37°, washed and resuspended at 10^5 cells/ml. Individual spleen cells from control and infected mice were used as effector cells. Effector and target cells (cell ratio 100:1, 50:1 and 25:1) were placed in U-shaped 96-well microtitre plates (Flow Laboratories) in a total volume of 0·2 ml. After 4 hr incubation at 37°, the specific ^{51}Cr release was calculated as follows:

$$\% \text{ specific } {}^{51}\text{Cr release} = \frac{\text{test c.p.m.} - \text{baseline c.p.m.}}{\text{total c.p.m. incorporated} - \text{baseline c.p.m.}} \times 100.$$

Tests were run in quadruplicate and baseline release never exceeded 10% of total counts incorporated by target cells.

T-cell enrichment

T cells were enriched by the direct 'panning' method (Wysocki & Sato, 1978). Briefly, polystyrene petri-dishes (Flow Laboratories) were coated with antibody specific for mouse Ig (Becton-Dickinson, Mountain View, CA). 20×10^6 spleen cells from infected or control mice were added and allowed to adhere to coated plates for 90 min at 4°. Non-adherent cells were harvested, washed and resuspended in culture complete medium. Cell viability was >95% (tryplan blue exclusion). T-cell recovery, determined by FACS, was ranging from 90% to 95%. The adherent population, tested by FACS, contained a majority of Ig^+ cells.

Macrophage isolation

Spleen cells were placed in 100-mm plastic culture dishes (Flow Laboratories), coated in advance with inactivated FCS by overnight incubation at 4°, and incubated for 1 hr at 37° in 5% CO_2. Non-adherent cells were harvested, washed and resuspended in RPMI complete medium. The resulting monolayers were overlayed with a medium containing 0·02% EDTA. After 20 min incubation at 37°, the adherent population (namely macrophages) was harvested by vigorous pipetting, washed and resuspended in complete RPMI medium.

T-cell Lyt-2$^+$ depletion

1×10^7 spleen lymphocytes from infected and control mice were resuspended in RPMI-1640 medium containing 1:20 dilution of Lyt-2.2 antiserum (Cederlane Laboratories, Hornoby, Ontario, Canada) and incubated at 4° for 45 min. After incubation, the cells were washed, and incubated in a culture medium containing 1:10 dilution of low toxic rabbit complement (C) (Cederlane) for 1 hr at 37°. Spleen cells, incubated with either the medium alone or with a 1:10 dilution of rabbit complement, were used as control. After the end of incubation the cells were washed and resuspended in RPMI complete medium. These cell suspensions (analysed by FACS) contained less than 0·6% of a Ts subpopulation. The viability was >90%.

Statistical analysis

Statistical analysis was performed with Student's *t*-test.

RESULTS

Inhibition of IL-2 production by spleen cells from PR8-infected mice

These studies arise from the observation that the influenza virus infection affected the proliferative responses of T lymphocytes in mice (Del Gobbo *et al.*, 1985). In order to analyse this impaired response, we tested whether the PR8 virus infection modulates IL-2 production on mitogen-stimulated splenocytes. At Days 1, 2, 4, 6, 8, 10 and 14 after infection, spleens from control and infected mice were removed and assayed for IL-2 production upon *in vitro* stimulation (Fig. 1). Data show that the PR8 virus infection significantly reduced IL-2 production and that this decrease is virus dose-dependent. In fact, in mice infected with 1×10^4 CPE 50% units (Fig. 1b) these effects were evident 1 day after infection and reached the maximum at later stages (4 days), while mice inoculated with 5×10^3 CPE 50% units (Fig. 1a) showed significant IL-2 reduction later (on 8 days), and this effect was shorter.

The same IL-2 inhibition pattern was observed when spleen cells from infected mice were stimulated *in vitro* with inactivated PR8 influenza virus, at Days 1, 4, 6 and 8 post-infection (data not shown).

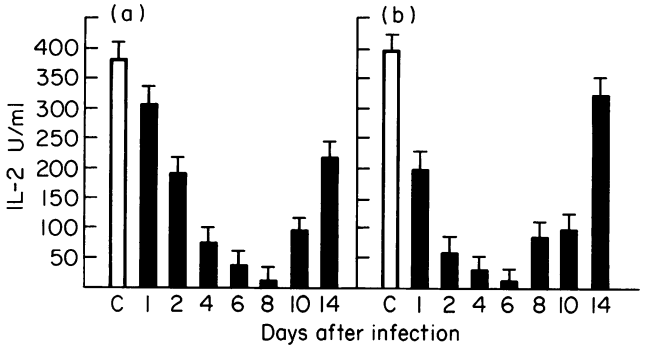

Figure 1. IL-2 activity in BALB/c mice, infected with 5×10^3 CPE 50% units (a) and 1×10^4 CPE 50% units (b) and in control mice (c) at Days 1, 2, 4, 6, 8, 10 and 14. IL-2 production was assayed, according to the probit analysis method of Sette *et al.* (1986). IL-2 activity in spleen cells from control and infected mice is expressed in U/ml. The reported values represent the means of individual spleen cell IL-2 production (10 mice for each experimental group). Each experiment was repeated three times. Statistical analysis was performed with Student's *t*-test ($P < 0.05$).

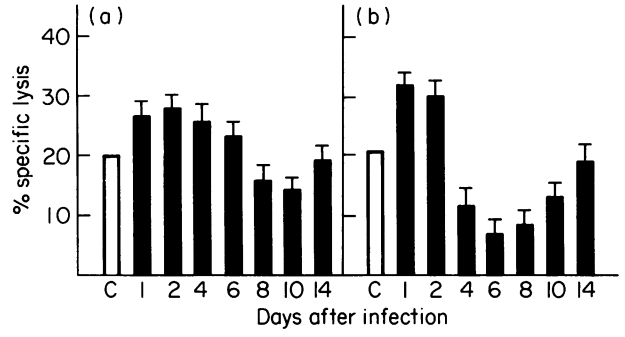

Figure 2. NK cell cytolitic activity in BALB/c mice (infected with same doses as shown in Fig. 1) at effector : target cell ratio 100 : 1. On Days 1, 2, 4, 6, 8, 10 and 14, control and infected mice were killed and individual spleen cell NK activity was tested. The results represent the means of individual spleen cell NK activity of 10 animals for each experimental group. Each experiment was repeated three times. Statistical analysis was performed with Student's *t*-test ($P < 0.001$).

Modulation in NK cell activity

This result indicates that the influenza virus infection produces an impairment of T-helper cell functions. Therefore we examined whether the loss of IL-2 production may modulate the natural immune response. Spleen cells from control and infected mice were assayed for NK cell activity at equal time intervals (Fig. 2). The results confirm this hypothesis. In fact, after a transient increase, the NK cell activity was significantly reduced in both groups of virus-infected mice and the minimum ^{51}Cr release appears to be correlated with the lower IL-2 production. These results point out that influenza virus infection causes an impairment of immune functions, through a reduced IL-2 production by T-helper cells.

T-cell enrichment does not increase IL-2 production levels

In order to explain the suppressive effect on IL-2 production, we carried out a number of experiments for which we chose the highest virus dose (1×10^4 CPE 50% units) and the day when the immunosuppressive effect was most evident (Day 6). As a first working hypothesis, we tested whether the decrease in IL-2 production depends upon a T-cell number reduction. In fact, during the course of infection we observed that the spleen weight and cell phenotypes were significantly alterated. Spleens did not show any necrosis and their weights were decreased on Day 6 post-infection (0·06 g compared with 0·14 g of control mice) ($P < 0.001$). On the same day, Thy-1.2, L3T4 and Ly 2 positive spleen cells were tested by FACS analysis, using fluoresceinated monoclonal antibodies (Becton-Dickinson). Spleens from infected mice showed 38·3% ± 2·5 Thy-1.2-positive cells (control 42·6% ± 2·0), 19·2% ± 1·7 L3T4-positive cells (controls 26·0% ± 3·0) and 17·3% ± 1·0 Ly 2-positive cells (controls 13·6% ± 1·3). For this purpose, we enriched a T-cell subpopulation by the direct panning technique. Figure 3 shows the IL-2 production in control and infected mice before and after T-cell enrichment. The results show that a T-cell reduced number is not responsible for defective IL-2 production. In fact, the T-cell enrichment was unable to restore normal levels of IL-2 production by spleens of infected mice. The adherent cells (namely B lymphocytes) produce no measurable amount of IL-2 (data not presented).

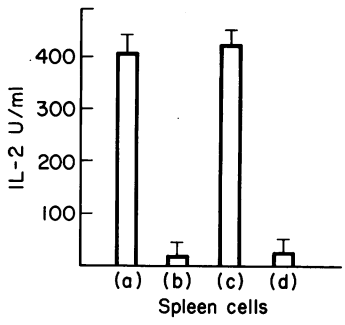

Figure 3. Influence of T enrichment on the IL-2 production by splenocytes from control and PR8 virus-infected BALB/c mice (1×10^4 CPE 50% units) at Day 6. (a) Control unfractionated spleen cells; (b) PR8 virus-infected unfractionated spleen cells; (c) control panning-enriched T cells; (d) PR8 virus-infected panning-enriched T cells. IL-2 production was assayed, according to the probit analysis method of Sette *et al.* (1986). IL-2 activity in spleen cells from control and infected mice is expressed in U/ml. The reported values represent the means of individual spleen cell IL-2 production (10 mice for each experimental group). Each experiment was repeated three times. Statistical analysis was performed with Student's *t*-test ($P < 0.05$).

Macrophages are not involved in the decrease of IL-2 production

To determine whether macrophages are involved in the defective IL-2 production (through an altered IL-1 production or by antigen-defective presentation) (Larsson, Iscove & Coutinho, 1980; Smith *et al.*, 1980), we removed macrophages from control and infected spleen cells and then performed the following mixed experiments. Macrophages pooled from control spleen cells were co-cultured with the infected non-adherent spleen cells, and vice versa. Macrophages were co-cultured with non-adherent spleen cells at ratio 1 : 5, because preliminary dose–response studies established that this concentration provides maximum synergism (data not presented).

Figure 4 shows the levels of IL-2 production in all combinations. The results demonstrate that macrophages are not

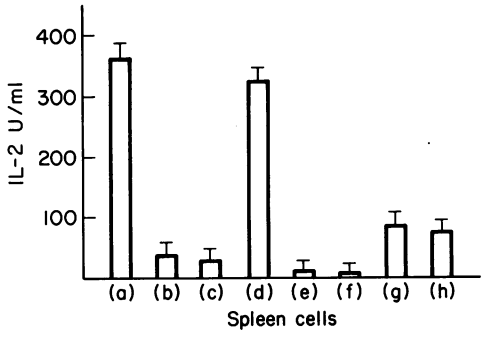

Figure 4. Interaction of adherent cells and non-adherent cells in IL-2 production by spleen cells from control and PR8 virus-infected BALB/c mice (1×10^4 CPE-50% units, at Day 6). (a) Control unfractionated spleen cells; (b) infected unfractionated spleen cells; (c) control adherent cells + infected non-adherent cells; (d) infected adherent cells + control non-adherent cells; (e) control adherent cells only; (f) infected adherent cells only; (g) control non-adherent cells only; (h) infected non-adherent cells only. IL-2 production was assayed according to the probit analysis method of Sette *et al.* (1986). IL-2 activity in spleen cells from control and infected mice is expressed in U/ml. The reported values represent the means of individual spleen cell IL-2 production (10 mice for each experimental group). Each experiment was repeated three times. Statistical analysis was performed with Student's *t*-test ($P < 0.05$).

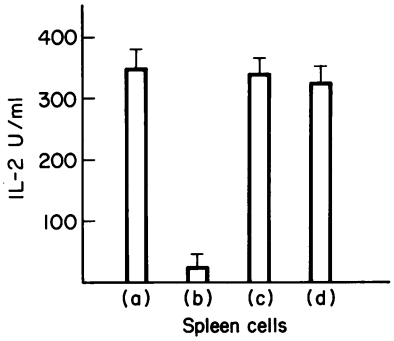

Figure 5. Effect of anti-Lyt-2.2 and C treatment on IL-2 production by control and PR8 virus-infected BALB/c mice (1×10^4 CPE 50% units, at Day 6). (a) Control untreated spleen cells; (b) infected untreated spleen cells; (c) control anti-Lyt-2.2 + C-treated spleen cells; (d) infected anti-Lyt-2.2 + C-treated spleen cells. IL-2 production was assayed according to the probit analysis method of Sette *et al.* (1986). IL-2 activity in spleen cells from control and infected mice is expressed in U/ml. The reported values represent the means of individual spleen cell IL-2 production (10 mice for each experimental group). Each experiment was repeated three times. Statistical analysis was performed with Student's *t*-test ($P < 0.05$).

involved in defective IL-2 production. In fact control macrophages co-cultured with infected non-adherent cells did not reverse the impaired IL-2 levels and macrophages from infected mice were unable to suppress IL-2 production by control non-adherent cells. The cultures of non-adherent cells and macrophages alone from control and infected mice produced no measurable levels of IL-2.

T-suppressor cell depletion is able to restore normal levels of IL-2 production by spleen from virus-infected mice

Since it is shown that T suppressor (Ts) cells play an important regulatory role in the balance of normal immune response (Gershon, Liebhaber & Ryu, 1974), we considered the possibility that the fall of IL-2 production may be due to Ts cells. In order to examine this hypothesis, the Ts cell subpopulation was removed from control and infected spleen cell suspensions (by anti-Ly 2.2 antisera + complement). The levels of IL-2 production before (a) and after (b) the Ts depletion are shown in Fig. 5. The results point out that infected splenocytes are able to produce normal levels of IL-2 after Ts removal, demonstrating that this T-cell subpopulation is responsible for the decreased IL-2 production observed in PR8-infected mice.

DISCUSSION

The experiments described in this report help to shed light on the nature of immunosuppression observed in influenza virus-infected hosts. The results demonstrate that the PR8 virus infection significantly affects IL-2 production, supporting the central role of this interleukin in the regulation of immune response. In addition, we report that the impaired IL-2 production could produce in turn an inhibition of NK cell activity in spleens from infected mice. In fact we showed that, in both groups of infected mice, the minimum NK cell activity appeared to be correlated with the lower levels of IL-2 production. This is in agreement with data from other authors, describing the defect of NK cell activity associated with a decreased IL-2 production (Hanney *et al.*, 1981; Kabelitz *et al.* 1985). This immune function impairment is virus dose-dependent and related to virus replication, according to the following: (i) good time correlation between the impairments and the two virus doses used; and (ii) absence of any change in mice inoculated i.n. with UV-inactivated viruses (which completely destroy viral infectivity) (data not shown). Low IL-2 production has been reported in a number of infections (Blackett & Mims, 1988; Wainberg *et al.*, 1985), but very little is known about the mechanism. As a working hypothesis we investigated whether the IL-2 production can be due to a primary deficiency on IL-1 production. The results of cell-mixing experiments indicate that control non-adherent cells co-cultured with infected adherent cells (namely macrophages) produce normal levels of IL-2, showing that the IL-1 production was not involved in this phenomen. As another working hypothesis we studied whether these lower levels of IL-2 production were related to a decreased T-cell number, as observed in infected mice. Our data show that PR8-infected splenocytes, enriched in T cells (by panning technique), were not able to produce normal levels of IL-2. The present results point out that the decreased IL-2 production, observed in PR8-infected mice, appears to be related, at least in part, with T suppressor cells. In fact, when the Ts cell subpopulation was removed (by anti-Ly 2 + C) from infected spleen cells, we observed normal levels of IL-2 production.

The ability of T-suppressor cells to modify T-helper cell (Th) functions is well described. Recent studies demonstrate that Ts cells inhibit cellular DNA synthesis and IL-2 production by a specific or non-specific factors or by cell–cell contact (Chiba, Nishimura & Hashimoto, 1985; Chouaib *et al.*, 1984; Fisher & Swierkosz, 1987; Kramer & Koszinowski, 1982).

The mechanism of Ts cell induction *in vivo* during influenza virus infection remains to be explained. One possible hypothesis is that the PR8 virus stimulates directly the Ts cell subpopulation. Some reports described influenza-induced suppressor cells,

which inhibit a variety of T-cell functions (Hurwitz & Hackett, 1985; Liew & Russel, 1980). Another explanation for an increased number of Ts cells in infected mice is that PR8 viruses can preferentially infect and replicate in T-helper cell subsets. Many reports show that paramyxovirus and myxovirus infect blood lymphocytes and, in particular, the T-helper cell subpopulation (Casall, Rice & Oldstone, 1984; Jacobson & McFarland, 1984). This hypothesis is in agreement with our data showing that during the course of a PR8 virus infection the percentage of L3T4-positive cells is significantly reduced. The two previous hypotheses are not necessarily in contrast; in fact PR8 viruses, at the same time, can both stimulate the Ts cell subset and infect Th cells.

In conclusion, PR8 virus infection affects IL-2 production and NK cell activity. This impairment appears to be related to an increased number of the Ts cell subset. Its mechanism remains to be explained.

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