Brief Definitive Report

IN VITRO MACROPHAGE MANIFESTATION OF CORTISONE-INDUCED DECREASE IN RESISTANCE TO MOUSE HEPATITIS VIRUS*

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It has been known since cortisone was first used that acute infections are often more severe when this drug is administered and that a number of immune responses are suppressed by this drug (1). We show here that peritoneal exudate cells (PEC) taken from hydrocortisone (HC)-treated, normally resistant C_3H mice are 1,000 times more susceptible to challenge with mouse hepatitis virus (MHV) in vitro than are PEC from untreated mice. Furthermore, resistant macrophages from normal C_3H mice succumb to viral destruction when cultured with spleen cells from cortisone-treated mice of the same strain but not with spleen cells of normal mice of the same strain.

Materials and Methods

Animals. 4–6-wk-old inbred C_3H and C_3H_{SS} mice were used. The C_3H mice are resistant to mouse hepatitis virus (MHV-2) and have been maintained by continuous inbreeding in this laboratory since 1955. The C_3H_{SS} strain of mice, into which the gene for susceptibility was introduced, was developed by crossing susceptible PRI mice with C_3H mice and back-crossing the hybrids with the C_3H. The offspring were selected by testing the susceptibility of macrophages from individual mice and then breeding from those that had susceptible cells. The congenic strain derived from this is just as susceptible as the PRI mouse (2).

Virus. The MHV-2 strain of mouse hepatitis virus originally obtained from Dr. J. Nelson, Rockefeller Institute, Princeton, N. J. in 1955, has been maintained in this laboratory by passage through 1-mo-old PRI mice.

Culture of PEC. Methods of harvesting and culturing peritoneal macrophages have been previously described in detail (3). PEC containing >90% macrophages were resuspended in Chang's medium and then dispensed into 12- × 100-mm Wassermann tubes. All tubes were incubated at 37°C in a roller drum.

HC Treatment. Mice were injected i.p. with 3 ml of thioglycollate medium on day 0, followed 12 h later by 2.5 mg HC (Solu-Cortef; Upjohn Co., Kalamazoo, Mich.). Two more injections of 2.5 mg HC were given 12 and 36 h after the first dose. PEC were harvested on day 3 (i.e., 24 h after the last dose). Control mice received phosphate-buffered saline instead of HC.

Spleen Cells from Mice with Cortisone Treatment. Mice were given 2.5 mg of cortisone on day 0; on day 3, they were killed and spleen cells were released by gentle teasing. 0.5×10^6 spleen cells were cultured together with normal C_3H macrophages for 24 h. For control, equal numbers of syngeneic spleen cells from untreated mice were used.

Preparation of Supernatant Fluid from concanavalin A (Con A)-treated Spleen Cells. Details of the preparation of supernatant fluid from Con A-treated spleen cells are provided elsewhere (4). Briefly, spleen cells from Con A-treated mice were cultured for 3 d in the presence of 2 μg/ml of Con A. Supernatant fluid was obtained by centrifuging the cell suspension at 1,000 rpm for 30 min.

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Results

Effect of Adding Spleen Cells from Cortisone-treated Mice. Previous work from our laboratory has suggested that susceptibility of macrophages may be regulated by various lymphokines. Therefore, cells from cortisone-treated mice were combined in vitro with normally resistant C_3H macrophages and the susceptibility of these was tested. C_3H mice were given 2.5 mg cortisone on day 0, and the spleens were removed 3 d later. 0.5×10^6 spleen cells were then added to each culture of normal C_3H macrophages. Control macrophage cultures received equal numbers of untreated spleen cells from syngeneic mice. Various dilutions of virus [MHV(PRI)] were inoculated into both groups of cultures. All cultures were kept for 6 d at 37°C. The results are summarized in Table I. Genetically resistant macrophages were destroyed by day 3 at 100-fold greater dilutions when they were cocultivated with spleen cells from cortisone-treated spleen cells but not the normal spleen cells. Spleen cells that had been killed by heating to 56°C for 30 min or by freezing and thawing did not alter the resistance of C_3H macrophages.

Direct Addition of HC to Peritoneal Adherent Cell Populations. 10, 50, 100, 150, or 350 μg of HC was added to normal C_3H macrophages in culture and incubated for a period of 24 h before viral infection. The mean lethal dose (LD_{50}) of the virus in these macrophages was found to be $10^{2.5}$ for control macrophages (i.e., no HC), $10^{3.0}$, $10^{3.0}$, $10^{3.5}$, and $10^{2.8}$ when 10, 50, 100, and 150 μg of HC was given, respectively. None of the cultures inoculated with these amounts of HC alone showed destruction, but 350 μg of HC was toxic to the macrophages.

In Vivo Induction of In Vitro Susceptibility of C_3H Macrophages to MHV(PRI) Virus. Earlier work had failed to induce large changes in susceptibility of macrophage cultures even though it was always clear that the mouse was made highly susceptible with cortisone. Table II, however, shows that macrophages from HC-treated resistant mice (which had been treated three times) were 400–1,000 times more susceptible to the virus than those from untreated mice. When the mice were given only one injection of HC, the macrophages were not susceptible. Viral growth was demonstrated primarily by cell destruction, which occurred as early as 2 d, but giant-cell formation also occurred which eventually ended in loss of contact with the culture tube. Virus yield was determined in critical cases. The 1,000-fold increase in susceptibility of the macrophages was routinely observed if three doses of HC ranging from 50 μg to 5 mg were given to the mice, but when 10 mg was used, the cells were only 10-fold more susceptible.

Shif and Bang (3) demonstrated that when high multiplicities of MHV(PRI) were

TABLE I

Effect of Adding Spleen Cells from Cortisone-treated Mice to Cultures of Syngeneic Macrophages

Virus	Source of spleen cells*	
	Cortisone-treated mice	Untreated syngeneic mice
10^{-1}	6/6	6/6
10^{-2}	6/6	4/6
10^{-3}	6/6	0/6
10^{-4}	5/6	0/6
10^{-5}	0/6	0/6

* 0.5×10^6 spleen cells were added to each macrophage culture.

TABLE II

Susceptibility of C_3H Macrophages to MHV(PRI) After In Vivo Administration of HC

C_3H* control	1 mg HC	2.5 mg HC	5 mg HC	10 mg HC	2.5 mg‡ HC	C_3H_{SS}* control
			$\log_{10}$ LD_{50}/0.1 ml			
2.4	4.6	5.4	4.8	3.5	2.6	7.7

Macrophage cultures were infected with serial 10-fold dilutions of MHV(PRI). Readings were taken 5 d postinfection. Figures represent dilution of virus producing destruction.

* Control mice received saline instead of HC.

‡ Single dose.

TABLE III

*Effect of Various Steroids on Resistance of Thioglycollate-elicited C_3H Macrophages**

C_3H control	Dexamethasone	Prednisolone	Progesterone	Testosterone	HC
		$\log_{10}$ LD_{50}/0.1 ml			
2.4	4.8	5.4	1.3	1.5	4.8

Each group of C_3H mice (six per group) received three intraperitoneal doses (1 mg each) of the steroid after thioglycollate stimulation, and PEC were harvested 24 h after the last dose.

* All macrophages in the experiment were seeded in 16-mm 24-well tissue culture cluster plates and incubated at 37°C with 5% CO_2 and humidified air.

given to C_3H macrophages, a mutant virus, MHV(C_3H), emerged that grew to high titer in both resistant and susceptible macrophages. Following the above findings, macrophage cultures from HC-treated mice receiving 10^4 LD_{50} of MHV(PRI), and which were destroyed by the virus, were assayed for virus yield on C_3H and C_3H_{SS} macrophages on days 5 and 7 postinfection. Virus harvested from macrophages of HC-treated mice on day 5 postinfection titered 10,000 times higher in C_3H_{SS} macrophages than in C_3H macrophages. By day 7 postinfection, the difference had decreased to 2 logs, presumably because of late growth of the mutant. Because of this, most of the subsequent experiments were limited to observations within 5 d of inoculation.

The effect of cortisone is apparently glucocorticoid-specific. Prednisolone and dexamethasone were administered in the same way as HC: three successive injections, intraperitoneally, in vivo. Two steroids (progesterone and testosterone) were similarly administered. The results showed that the active glucocorticoids increased susceptibility of the C_3H macrophages, whereas the other steroids did not (Table III).

Growth Curve of MHV(PRI) in Macrophages from C_3H_{SS}, C_3H, and HC-treated Mice. Virus (1.5×10^4 tissue culture infective doses/2×10^6 cells) was adsorbed for 60 min at 37°C in a roller drum. The fluid was then replaced by culture medium. At various intervals, individual tubes were harvested, frozen, then titered for total cellular and supernatant virus.

As can be seen from Fig. 1, there was a sharp increase in virus titer in C_3H_{SS} macrophages during the first 10 h. There was no rapid initial rise in virus titer in either normal or HC-treated C_3H macrophages. However, after about 11 h, the growth rate in the macrophages from HC-treated mice increased rapidly. At 21 h postinfection, virus replication in C_3H resistant macrophages started to decelerate, whereas in macrophages from HC-treated C_3H mice, it continued to increase. Although virus growth in the latter attained almost the same final yield as in C_3H_{SS}, it required ~20 h longer for this to occur.

In vivo, however, C_3H mice challenged with virus 3 d after cortisone (2.5 mg s.c.) die in just as short, or shorter, time period than susceptible C_3H_{SS} mice (mean survival time: 1.9 d for C_3H; 2.5 d for C_3H_{SS}). Earlier, Willenborg (7) had found that C_3H

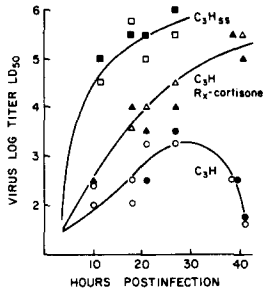

FIG. 1. Growth of MHV(PRI) in macrophages from C_3H_{SS}, cortisone-treated (R_x-cortisone) C_3H mice and untreated C_3H mice. Two independent experiments are shown for C_3H_{SS} (■, □), cortisone-treated C_3H (▲, △), and untreated C_3H (●, ○). LD_{50} were determined according to the method of Reed and Muench (6).

mice challenged with virus 1 h after cortisone administration take longer to die than genetically susceptible PRI mice. Evidently, the time of cortisone administration may be a factor in the induction of susceptibility to the virus.

Reversal of HC-induced Modulation by Addition of Supernate from Con A-treated Spleen Cells. It has been previously shown in our laboratory that genetically susceptible mice, as well as their macrophages, gain resistance when the mice are treated with Con A, or when the macrophages are treated with the supernate from Con A-treated spleen cells (4). It was therefore of interest to determine whether Con A would be antagonistic to cortisone.

Therefore, 0.1 ml of supernate fluid from Con A-treated spleen cells (4) and also from normal spleen cells was added to macrophages from HC-treated mice and incubated for 14 h before inoculation of virus. Table IV shows the complete reversal of the HC effect by supernatant fluid from Con A-treated spleen cells but only a partial reversal by supernate fluid from normal spleen cells.

The addition of normal spleen cells did not make the macrophages from cortisone-treated mice resistant again. 0.5×10^6 spleen cells/ml, harvested from normal C_3H mice, were added to macrophages from HC-treated mice and incubated for 24 h before viral infection. There was no significant difference between macrophages that received the spleen cells and those that did not (Table IV).

Discussion

Although it has been known almost from the start of research on cortisone that this powerful anti-inflammatory drug depresses various immunologic mechanisms (1), the way in which it alters susceptibility to specific agents is not clear. The correspondence between in vivo and in vitro susceptibility of macrophages to mouse hepatitis made this model an attractive one to study. Cortisone-induced increase in susceptibility of mice to mouse hepatitis virus has also been observed by a number of other investigators as summarized by Vella and Starr (8). Administration of cortisone to the genetically resistant C_3H mice (9), as well as to outbred mice, rendered them susceptible to viral [MHV(PRI)] infection. The LD_{50} of MHV(PRI) virus was $10^{8.5}$ in PRI mice, $10^{6.5}$ in cortisone-treated C_3H mice, and $<10^{1.0}$ in untreated C_3H mice (9). The slight or equivocal change in macrophage resistance, which had been demonstrated in vitro (7, 9), was in no way comparable to the marked effects on the intact animal. Gallily et al. (9), using a 1:100 dilution of stock virus (10^6 LD_{50}), had found that 85–100% of

TABLE IV

Effect of Adding Normal Spleen Cells or Supernatant Fluid from Con A-treated C_3H Spleen Cells to Macrophages from HC-treated Mice (HC Macrophages)

C_3H control macrophages	HC macrophages	HC macrophages and untreated spleen cells	HC macrophages and Con A supernate	HC macrophages* and normal spleen cell supernate
		log_{10} LD_{50}/0.1 ml		
2.3	5.7	5.5	1.5	3.5

0.5 × 10^6 spleen cells or 0.1 ml of Con A-activated spleen supernate was added 24 and 14 h before viral infection, respectively.

* Normal spleen cell supernate was obtained from a 96-h culture of normal C_3H spleen cells.

cortisone-treated cultures were killed by virus as compared to ~20% of cultures given virus alone. Willenborg (7), using a single dose of 2.5 mg cortisone, had found that the LD_{50} of MHV(PRI) virus in macrophage cultures from cortisone-treated C_3H mice was $10^{3.4}$, and in cultures from untreated mice, it was $10^{3.2}$. In our studies, it was apparently crucial to administer three doses of HC to the mice to achieve sufficient suppression of viral resistance for in vitro expression of the effect. Presumably, a 1,000-fold increase of susceptibility of macrophages may explain most, if not all, of the $10^{5.5}$ difference in titer in normal and cortisone-treated mice.

The mechanism whereby HC or cortisone changes the resistance of the all-important macrophage remains unclear. Addition of spleen cells from cortisone-treated mice enhanced the susceptibility of C_3H macrophages 100-fold. Furthermore, cultures from HC-treated mice remained susceptible up to 72 h in culture, but fully regained resistance by 1 wk. The implication is that resistance of C_3H macrophages is altered by some product of lymphoid cell activity induced by the steroid. That two other steroids, testosterone and progesterone, failed to cause the development of susceptible macrophages, whereas dexamethasone and prednisolone did, again suggests a correspondence of the in vitro results with the susceptibility of the whole animal.

The addition of whole spleen cells from normal resistant mice to macrophages from HC-treated mice for a period of 24 h before viral infection did not restore resistance. However, adding 0.1 ml of supernate fluid from Con A-stimulated spleen cells reversed the HC effect. This suggests that resistance is dependent, in part at least, upon a lymphokine released from T cells. Because Con A stimulates a variety of lymphokines, such as migration-inhibitory factor, interferon, soluble immune response suppressor, and T cell growth factor, it will be necessary to identify which specific lymphokine is responsible for the effect.

The growth-curve experiment reveals that during the first 12 h of observation, virus replication was equal in both sets of C_3H macrophages, but that after this period, virus growth started to decelerate in the C_3H control macrophages, although it continued to increase in macrophages from HC-treated mice. This suggests that resistance might be a result of the capacity of the C_3H macrophage to produce an anti-viral substance during early growth which restricts viral growth.

The work of Gillis et al. (10) on how cortisone is able to suppress immune responses begins to explain the well known ability of cortisone to suppress antibody and T cell response. Suppression of the production of a T cell growth factor by this corticosteroid is then followed by a failure of the T cell compartment to expand after either specific antigenic stimulus or administration of Con A. That Con A and cortisone act

antagonistically in their studies (10) is very relevant to the similar blocking effect of one by the other in our system. However, because our studies seem to demonstrate that the cortisone-treated cells cause a change in the macrophage rather than prevent the release of a factor from lymphocytes, the analogy needs to be explored further.

Summary

Genetically resistant C_3H mice routinely yielded macrophages that were resistant when grown in 90% horse serum. These mice also routinely yielded macrophages that were susceptible to the same virus, MHV(PRI), in vitro after the mice had been treated with three intraperitoneal doses of hydrocortisone. Dexamethasone and prednisolone when similarly administered also increased the susceptibility of C_3H macrophages taken from the treated animal, but progesterone and testosterone did not. In addition, spleen cells from mice treated with cortisone made the resistant C_3H macrophages 100 times more susceptible in vitro.

Increased in vitro susceptibility induced in this way by hydrocortisone was reversed by exposure to supernatant fluid removed from cultures of concanavalin A-treated spleen cells.

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