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Acute Antigen-Induced Elevation of Serum Colony Stimulating Factor (CFS) Levels

D. METCALF

Cancer Research Unit, Walter and Eliza Hall Institute, Post Office, Royal Melbourne Hospital, Victoria 3050, *Australia*

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Summary. Injection of endotoxin or *Salmonella* flagellins to C57BL mice caused 50–100-fold elevations, maximal at 3–9 hours, in the capacity of the serum to stimulate granulocyte and macrophage colony formation by mouse bone marrow cells in agar cultures. This response was not elicited by a variety of foreign serum proteins. The response was radioresistant but preimmunized mice showed a specific depression of responsiveness on challenge with the same antigen.

INTRODUCTION

Bone marrow progenitors of macrophages and granulocytes can proliferate in agar cultures to form large colonies of differentiating macrophages and/or granulocytes if the cultures are stimulated by the colony stimulating factor (CSF) (Bradley and Metcalf, 1966; Metcalf, Bradley and Robinson, 1967a). CSF appears to be a glycoprotein of molecular weight 45,000 (Stanley and Metcalf, 1969, 1971) which is demonstrable in the serum and urine of normal humans and mice (Robinson, Metcalf and Bradley, 1967; Foster, Metcalf, Robinson and Bradley, 1968a; Metcalf and Stanley, 1969) and appears to be a humoral regulator of granulopoiesis and macrophage formation. Serum levels of CSF were shown to become elevated in mice and humans following viral or bacterial infections (Foster *et al.*, 1968a; Foster, Metcalf and Kirchmyer, 1968b; Metcalf and Wahren, 1968).

McNeil (1970a, b) and Metcalf (1971a) demonstrated that a variety of antigens, when injected into mice, stimulated the proliferation *in vivo* of the cells forming colonies *in vitro* and that the addition of antigens *in vitro* to cultures of unimmunized bone marrow cells potentiated the growth of granulocytic and macrophage colonies. Analysis of the latter phenomenon suggested that the *in vitro* effects of antigens on colony growth might be mediated by the stimulation of an increased endogenous production of CSF by the cultured bone marrow cells, and raised the possibility that the effects of antigen *in vivo* might also be mediated by CSF, rather than be a direct antigen-induced proliferation of these progenitor cells.

The present experiments were undertaken to determine the response of serum CSF levels in mice to the injection of endotoxin and various antigens.

EXPERIMENTAL METHODS

Mice

Mice used were 2–3-month-old males and females of the C57BL strain. This strain was chosen for convenience in performing serum CSF assays because of the low content of

lipoprotein inhibitors in C57BL serum which can inhibit colony formation *in vitro* and mask the detection of CSF (Stanley, Robinson and Ada, 1968).

Antigens

Unless otherwise specified, all antigens were injected intravenously in an injected volume of 0·2 ml. Two preparations of endotoxin (β-lipopoly-saccaride) from *E. coli* or *S. typhimurium* were kindly supplied by Dr C. Jenkins of the Department of Microbiology, University of Adelaide. Polymerized flagellin preparations from *Salmonella adelaide, S. waycross* and SL 871 were kindly supplied by Mr J. Pye and prepared according to methods previously described (Ada, Nossal, Pye and Abbot, 1964). Monomeric flagellin and a cyanogen bromide digest of flagellin were prepared according to Ada, Parish, Nossal and Abbot (1967).

Assay for serum CSF

Test mice were bled from the axilla under ether anaesthesia; clots were allowed to retract for 1–2 hours at 4° and sera removed following centrifugation and stored at −15° for assay.

All assays on serum for colony stimulating activity were performed using 1 ml cultures containing 75,000 bone marrow cells from normal 2–3-month-old C57BL mice. The technique and media used for bone marrow cultures have recently been described in detail (Metcalf, 1970).

Serial dilutions of serum samples from 1:1 to 1:54 were prepared in distilled water and replicate samples of 0·1 ml of each dilution were pipetted into 35 mm plastic Petri dishes (Falcon Plastics, Los Angeles). One millilitre portions of the agar-medium held at 37° and containing 75,000 bone marrow cells per ml were pipetted into each Petri dish using a Cornwall automatic syringe and the medium mixed throughly with the serum to be assayed before gelling occurred. Cultures were incubated at 37° for 7 days in a fully humidified atmosphere of 10 per cent CO_2 in air and colony counts were performed using a dissecting microscope at ×25. Colony sizes ranged from 50 to 2000 cells depending on the concentration of CSF in the test specimen. All assay runs included pooled serum standards of known colony stimulating activity to monitor for significant variations in the culture media or incubating conditions.

There is a sigmoid dose–response relationship between CSF concentration and colony numbers (Metcalf and Stanley, 1969) and for each serum, colony stimulating activity (CSF) levels were determined from the linear portion of the dose–response curve. In most cases serum dilutions in the range 1:6 to 1:54 stimulated colony numbers within the linear portion of the curve (5–100 colonies per culture).

Significant variability was encountered in serum CSF levels between different batches of normal C57BL mice used at different times during the 18 month interval in which these experiments were performed. This variability was due in part to variations in culture media but mainly to the occurrence of minor epidemics at various times in the different animal rooms housing the mice. Because of this variability, experiments were performed on individual batches of matched mice of the same age and housed in the same animal room and each experiment included animals used as positive and negative controls. It will be observed in the results to follow that the levels of CSF in positive and negative controls sometimes vary from one experiment to another, and only internal comparisons between the experimental groups in any one experiment are valid.

Irradiation

Mice were exposed to total body irradiation. X-rays were generated by a Philips RT 250 unit operated at 250 kV, 15 mA. H.V.L. was 0·8 mm copper and a focal skin distance of 50 cm with full backscatter conditions was used to irradiate mice at a dose rate of 127 R/min.

RESULTS

1. EFFECT OF ENDOTOXIN AND POLYMERIZED FLAGELLIN ON SERUM COLONY STIMULATING ACTIVITY

The prototype response of serum colony stimulating activity to the injection of antigens was observed using endotoxin prepared either from *E. coli* or *S. typhimurium*. A single intravenous injection of 5 μg of endotoxin (β-lipopolysaccaride from *E. Coli*) to normal mice caused a marked rise in serum colony stimulating activity (Fig. 1). Levels began

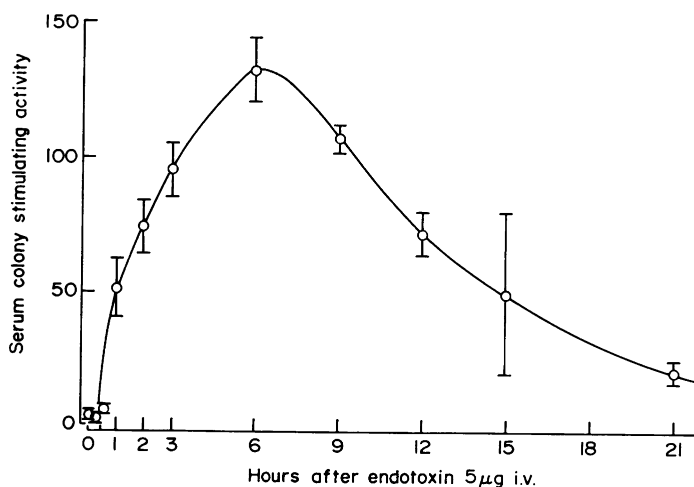

FIG. 1. Rise in serum colony stimulating activity in C57BL mice following a single intravenous injection of 5 μg endotoxin. Data are mean numbers of colonies stimulated by 0·1 ml of a 1:54 serum dilution. Each point mean value from five individual sera ± standard deviations.

rising 30 minutes after injection and were clearly elevated by 1 hour. The peak response occurred 3–9 hours after injection at which time serum colony stimulating activity was typically 50–100 times preinjection levels. Thereafter, serum activity fell rapidly and by 24 hours averaged only 2–5 times preinjection levels. A notable feature of this response was its high degree of uniformity. Of several hundred mice injected with endotoxin not a single mouse failed to respond by developing uniformly high elevations of colony stimulating activity maximal at 3–9 hours after injection.

A similar elevation of serum colony stimulating activity was observed following the intravenous injection of 20 μg of polymerized flagellin from *S. adelaide*. The peak rise occurred 3–6 hours after injection and levels returned to near normal by 24 hours after injection. The response to polymerized flagellin was smaller than with endotoxin, maximum rises of only 5–25-fold usually being obtained.

Because of the timing of the peak responses to endotoxin and polymerized flagellin, all subsequent assays on serum colony stimulating activity were restricted to sera taken 3 and/or 6 hours following antigen injection.

In Fig. 2 is shown serum colony stimulating activity in C57BL mice 3 hours after the intravenous injection of various doses of endotoxin or polymerized flagellin. Significant elevations of colony stimulating activity were obtained with 0·1 μg of endotoxin and 0·5 μg of polymerized flagellin. Polymerized flagellin appeared toxic for the mice, and when doses greater than 20 μg were used serum colony stimulating activity was lower than after the injection of smaller doses. Cortisone has been shown to rapidly lower serum CSF levels (Metcalf, 1969), and the lower responses observed with large doses of polymerized flagellin may represent the effects of adrenal-mediated stress.

Since residual amounts of endotoxin or flagellin would still be present in the serum of mice injected 3 or 6 hours previously, control experiments were undertaken to eliminate the

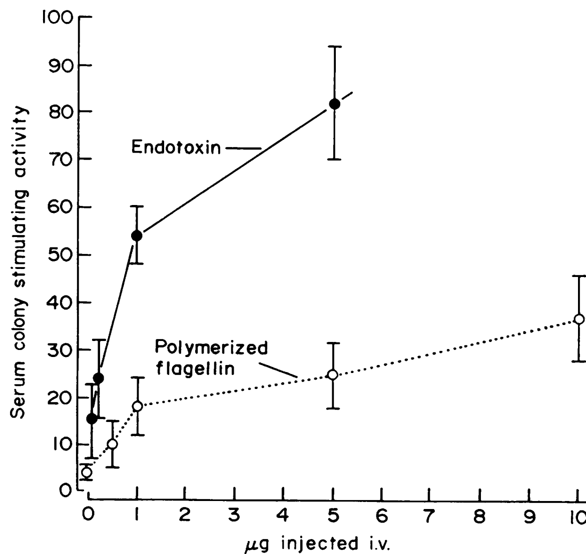

Fig. 2. Serum colony stimulating activity 3 hours following the injection of various doses of endotoxin or *S. adelaide* polymerized flagellin. Each point represents mean number of colonies stimulated by 0·1 ml of 1:54 serum from five individual mice ± standard deviations.

possibility that the elevated colony stimulating activity observed in the sera from these mice was due to the presence of residual amounts of the two antigens. Cultures of bone marrow cells were prepared containing 0·05 ml of undiluted pooled normal C57BL serum and 0·1–5 μg endotoxin or 0·1–20 μg polymerized flagellin. In agreement with previous studies (McNeill, 1970a; Metcalf, 1971) increased numbers of colonies were observed in such cultures compared with control cultures containing only C57BL serum. However the differences were slight (maximum increase in colony numbers of 50 per cent) compared with the extremely high colony stimulating activity of serum from mice injected either with endotoxin or flagellin. Preincubation *in vitro* of serum with either endotoxin or flagellin at 37° for 3 hours also failed to increase the colony stimulating activity of the serum above the minor increase of 30–50 per cent noted in the above control experiments. These experiments strongly suggested that the rises in serum colony stimulating activity observed following the injection of endotoxin or flagellin were not simply the result of

interactions between these materials and the plasma but involved some intermediate reaction with cells or tissues resulting in the production and/or release of CSF to the blood.

Previous studies (Metcalf *et al.*, 1967a) showed that after 7 days of incubation more than 90 per cent of bone marrow colonies which had been stimulated to develop by normal or leukaemic mouse serum were composed of pure populations of macrophages. It was a notable feature of the colonies stimulated by serum from mice injected with endotoxin or flagellin that at 7 days of incubation most colonies were either pure populations of granulocytes or mixed populations of granulocytes and macrophages (Table 1). This high proportion of granulocytic and mixed colonies persisted even when high dilutions of such sera, having no higher colony stimulating activity than normal undiluted C57BL serum, were used to stimulate colony formation.

TABLE 1
MORPHOLOGY OF COLONIES STIMULATED BY SERUM FROM MICE INJECTED WITH ENDOTOXIN

Serum Donor	Serum Dilution	Mean No. of colonies stimulated	Mean percentage of colonies*		
			Granulocytic	Mixed	Macrophage
Endotoxin-injected	1:2	152†	27	43	30
	1:6	114†	31	38	31
	1:18	72	26	61	13
	1:54	22	11	26	63
Normal	1:1	59	0	7	93

Each culture contained 75,000 C57BL bone marrow cells and 0·1 ml of the serum sample.
* Thirty sequential colonies sample from each type of culture.
† Colony counts on plateau portion of sigmoid dose–response curve.

2. FACTORS MODIFYING RESPONSE TO ENDOTOXIN OR FLAGELLIN

As shown in Table 2 the highest elevations of serum colony stimulating activity 3 hours after injection were obtained when endotoxin was injected by the intravenous route, the

TABLE 2
EFFECT OF ROUTE OF INJECTION AND OF PRE-IRRADIATION ON THE RESPONSE OF SERUM COLONY STIMULATING ACTIVITY TO ENDOTOXIN

Material injected*	Route of injection	Dose of irradiation (R)	Time of challenge after irradiation (hours)	Serum colony stimulating activity†
Endotoxin	i.v.	—	—	95 ± 5
	i.p.	—	—	55 ± 14
	s.c.	—	—	17 ± 8
Saline	i.v.	—	—	2 ± 1
Endotoxin	i.v.	0	24	51 ± 17
		600	24	72 ± 9
		800	24	83 ± 16
		1000	24	79 ± 19
		1200	24	83 ± 17
		1500	24	84 ± 16
Endotoxin	i.v.	850	0	63 ± 17
		850	24	56 ± 7
		850	48	52 ± 13

* Mice were injected with 5 μg endotoxin or 0·2 ml saline and serum collected 3 hours later.
† Six mice per group. Data are mean number of colonies stimulated by 0·1 ml of 1:54 dilution of serum ± standard deviation.

response being reduced when intraperitoneal or subcutaneous routes were used. Similar data were obtained for the response to polymerized *S. adelaide* flagellin. It may also be seen from Table 2 that the response to endotoxin was not prevented by prior whole-body irradiation of up to 1500 R, carried out either immediately before injection or up to 2 days previously.

The injection of cortisone acetate (1 mg) subcutaneously at the time of administration of endotoxin reduced the magnitude of the elevation of colony stimulating activity but this could have been due to the accelerated clearance of serum CSF to the urine which is known to follow the injection of cortisone (Chan, 1970) rather than to inhibition of the production or release of CSF.

Tests performed on C57BL mice aged between 1 week and 2 years indicated that mice at all ages could respond to injections of polymerized flagellin and developed equivalent elevations of serum colony stimulating activity 3 hours after injection.

3. Response to other antigens

As shown in Table 3, serum colony stimulating activity in mice injected 3 hours previously with monomeric flagellin from *S. adelaide* rose as high as in mice injected with

Table 3

Response of serum colony stimulating activity to various antigens injected intravenously

Expt No.	Antigen injected	Dose	Mean serum colony stimulating activity*
I	Polymerized flagellin (*S. adelaide*)	20 μg	97 ± 17
	Monomeric flagellin (*S. adelaide*)	20 μg	98 ± 17
	Flagellin digest (*S. adelaide*)	20 μg	22 ± 12
	Saline	0·2 ml	19 ± 5
II	Polymerized flagellin (*S. adelaide*)	20 μg	72 ± 9
	Polymerized flagellin (*S. waycross*)	20 μg	267 ± 33
	Polymerized flagellin (SL871)	20 μg	167 ± 56
	Saline	0·2 ml	3 ± 2
III	Sheep RBC	5×10^8	4 ± 2
	BSA	100 μg	5 ± 3
	HGG	100 μg	3 ± 2
	FGG	100 μg	4 ± 2
	TGAL	100 μg	8 ± 5
	Endotoxin (*E. coli*)	5 μg	98 ± 14
	Saline	0·2 ml	6 ± 4

* Five or six mice per group; serum collected 3 hours after injection. Data shown are mean number of colonies stimulated by 0·1 ml of 1:18 serum ± standard deviations.

polymerized flagellin. However, the injection of the nonantigenic cyanogen bromide digest of flagellin did not elicit any rise in serum activity.

Parallel tests on polymerized flagellins prepared from *Salmonella waycross* and SL 871 indicated that these antigens elicited even higher responses than did flagellin from *S. adelaide*. The injection of 0·05 ml of commercially prepared vaccines of *B. abortus*, pertussis and diphtheria toxoid elicited responses similar to those induced by polymerized flagellin. On the other hand, injections of xenogeneic red cells or allogeneic lymphoid cells, HSA, BSA, HGG, FGG and the synthetic antigen TGAL failed to elicit rises in serum colony stimulating activity when the sera were sampled 3 hours after injection.

4. RESPONSE TO REPEATED INJECTIONS OF ANTIGEN

Mice injected with polymerized flagellin were able to respond to a second or third intravenous injection at 24 hour intervals (Fig. 3), although the height and duration of the

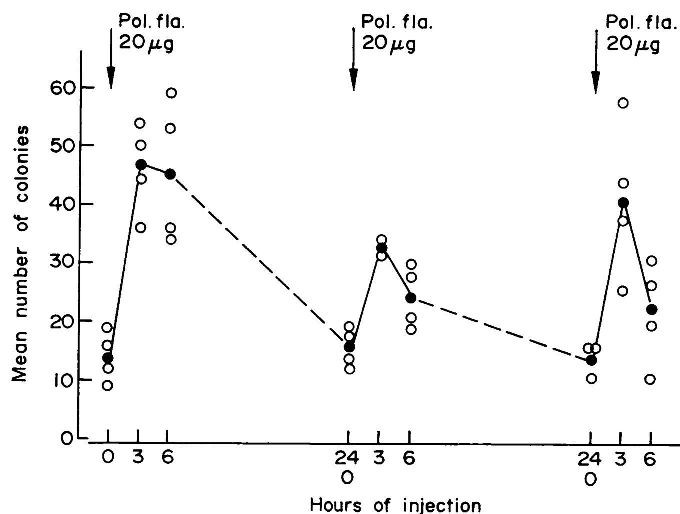

FIG. 3. Response of serum colony stimulating activity to second and third injections of 20 µg polymerized flagellin. Points represent mean numbers of colonies stimulated by 0·1 ml of 1:18 dilutions of sera from individual mice, solid circles are arithmetic means of group. All sera are from different mice, each mouse being bled only once.

subsequent responses appeared to be diminished. This prompted an examination of responses to challenge intravenous injections of polymerized flagellin in mice preimmunized with flagellin or preinjected with endotoxin or another polymerized flagellin.

In the first series of experiments mice were injected intravenously with 20 µg polymerized flagellin (*S. adelaide*), 5 µg endotoxin (*E. coli*) or 0·2 ml of saline, 3 weeks later these mice were injected again with the same dose, this time intraperitoneally. One week later, mice were challenged by an intravenous injection of polymerized flagellin or endotoxin and their sera collected for assay 3 hours after challenge. The results (Table 4)

TABLE 4
EFFECT OF PRE-IMMUNIZATION ON RESPONSE OF SERUM COLONY STIMULATING ACTIVITY TO CHALLENGE WITH ENDOTOXIN (*E. Coli*) OR POLYMERIZED FLAGELLIN (*S. adelaide*)

Immunization*	Challenge	Serum colony stimulating activity†	
		3 hours	6 hours
—	Saline	1±1	1±1
—	Endotoxin	110±28	131±15
—	Polymerized flagellin	35±6	21±3
Endotoxin	Endotoxin	16±12	14±8
Polymerized flagellin	Endotoxin	60±31	67±28
Polymerized flagellin	Polymerized flagellin	4±4	3±3
Endotoxin	Polymerized flagellin	21±3	18±4

* Injected i.v. with 5 µg endotoxin or 20 µg polymerized flagellin 4 weeks before and i.p. with the same dose 1 week before challenge.

† Six mice per group. Data shown are mean numbers of colonies stimulated by 0·1 ml of 1:18 serum ± standard deviations.

indicated that mice preinjected with either endotoxin or flagellin exhibited markedly diminished responsiveness when challenged with the same material. On cross challenge, some diminution in responsiveness was observed compared with responses in mice preinjected with saline, but responses were significantly higher than in mice being challenged with the same material injected during immunization.

Some uncertainty exists regarding antigenic cross-reactivity between polymerized flagellins prepared from different strains of *Salmonella*. Some appear not to be cross-reacting as assessed by the ability to elicit antibody formation but yet are cross-tolerogenic (Austin and Nossal, 1966). The results from cross-challenge experiments using three polymerized flagellins from *S. adelaide*, *S. waycross* and SL 871 gave similarly ambiguous results. In every instance, mice preimmunized with one flagellin exhibited markedly depressed responses when challenged with the same flagellin (Table 5). However, cross-

TABLE 5

EFFECT OF PRE-IMMUNIZATION ON RESPONSE OF SERUM COLONY STIMULATING ACTIVITY TO CHALLENGE AND CROSS-CHALLENGE WITH POLYMERIZED FLAGELLIN

Polymerized flagellin used for immunization*	Polymerized flagellin or other materials used for challenge	Serum colony stimulating activity†
Saline	Saline	5 ± 3
Saline	Endotoxin	63 ± 24‡
Saline	*S. adelaide*	70 ± 14
Saline	SL 871	83 ± 31
Saline	*S. waycross*	67 ± 36
S. adelaide	*S. adelaide*	14 ± 7
SL 871	SL 871	13 ± 4
S. waycross	*S. waycross*	17 ± 9
SL 871	*S. adelaide*	14 ± 3
S. waycross	*S. adelaide*	15 ± 10
S. adelaide	SL 871	33 ± 23
S. waycross	SL 871	32 ± 14
S. adelaide	*S. waycross*	56 ± 17
SL 871	*S. waycross*	63 ± 11

* Injected i.v. with 20 μg polymerized flagellin or 0·2 ml of saline 4 weeks before challenge and i.p. 1 week before challenge. Sera collected 3 hours after challenge.

† Six mice per group. Data shown are mean number of colonies stimulated by 0·1 ml of 1:18 dilution of serum ± standard deviations.

‡ Mean number of colonies stimulated by 1:45 dilution of serum.

challenge responses to *S. adelaide* and Sl 871 flagellin were depressed by preimmunization with one of the other flagellins, whereas crosschallenge with *S. waycross* flagellin elicited responses which were almost as high as in unimmunized control mice. In two repeat experiments using this particular design, identical results were obtained, and the data suggest that whilst flagellin from *S. adelaide*, *S. waycross* and SL 871 may share major antigenic determinants, *S. waycross* may possess an additional major determinant not present in either of the others.

DISCUSSION

The present experiments have shown that the injection of endotoxin or bacterial antigens to mice elicits a dramatic rise in the ability of the serum from such mice to stimulate colony formation *in vitro* by normal bone marrow cells. This rise is detectable in the serum

within 1 hour of injection, and parallel experiments in this laboratory have shown that even more rapid rises occur in the colony stimulating activity of extracts from various organs, e.g. thymus, spleen, submaxillary gland (Sheridan, J., personal communication). The response is relatively short-lived with serum activity returning to near-normal levels within 24 hours, although assays on urine from such animals have indicated that an increased excretion of CSF in the urine continues for more than 48 hours (Chan, S. H., personal communication). The injection of endotoxin to mice has proved to be a reliable system for obtaining high potency serum for analysing the initiation and growth of bone marrow colonies *in vitro*.

These observations document another recognition-type response to antigenic material although they have not localized the cells responsible for recognizing the antigenic material nor the mechanisms involved in the development of increased colony stimulating activity in the serum and tissues. The demonstration that serum CSF levels respond to antigens of this type complements earlier observations that serum CSF levels are unusually low in germfree mice (Metcalf, Foster and Pollard, 1967b) and that CSF levels rise after viral and bacterial infections. It seems reasonable to speculate that the CSF levels observed in normal mice in part may represent a response to the continual entry of antigenic material into the body, particularly of bacterial origin from saphrophytic organisms or subclinical infections. Since CSF appears to be a humoral regulator of granulopoiesis and monocyte formation *in vivo* it seems likely that the increased serum CSF levels developing in response to antigens of the above type may mediate the increased production of granulocytes and monocytes/macrophages known to occur after antigenic stimulation or bacterial infections.

The granulocytic nature of many of the colonies stimulated by the serum from mice injected with antigens is of some interest in view of the fact that normal serum always stimulates macrophage colony formation. It is possible that the 'reactive' CSF involved may differ from the normal CSF or that another factor may be present in such sera which modifies the action of CSF.

The high activity shown by endotoxin in eliciting rises in serum CSF levels may be related to its capacity to modify the response of animals to infections and antigenic stimulation, but complicates the interpretation of responses observed to bacterial antigens. There is always the possibility that such antigens are contaminated with small amounts of endotoxin and it could be argued that the responses observed to the flagellin were due to such contaminating endotoxin. In the case of the polymerized flagellin prepared from *Salmonella adelaide*, chemical estimations have shown that the endotoxin content of this preparation is less than 1·0 per cent (Ada *et al.*, 1964) and the dose–response data in the present experiments would require a contamination of at least 10 per cent to be explicable on the basis of endotoxin contamination. Thus it seems unlikely that endotoxin contamination can account for the responses noted to bacterial flagellin and the failure of the non-antigenic cyanogen bromide digest to elicit a rise in colony stimulating activity supports the conclusion that the rises are due to antigenicity of the preparations used. No explanation can be offered at present for the failure of foreign serum proteins or cells to elicit rises in serum CSF levels, although preliminary data have suggested that serum proteins conjugated with DNP sometimes elicit rises in serum colony stimulating activity (Metcalf, D. and Warner, N. L., unpublished data).

The depressed responsiveness of preimmunized mice to challenge injections is of interest, although these experiments need amplification, using bacterial antigens capable of

eliciting serum CSF rises but completely non-cross-reactive. Two mechanisms may be operating in such mice (a) a pharmacological-type state of depressed responsiveness following earlier responses to the immunizing injections of the antigens and/or (b) a specific depressed responsiveness based possibly on the presence of a particular type of circulating antibody. The present system has some obvious analogies with immediate hypersensitivity and desensitization but further experiments are required to determine the cause of the depressed responsiveness.

The acute nature of the rises in serum CSF elicited by antigens make it possible that antigens do not stimulate the proliferation *in vivo* of granulocyte and macrophage precursors by a direct action, but do so via the stimulation of high levels of endogenous CSF.

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