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Active suppression masks an underlying enhancement of antibody production *in vitro* by spleen cells from BCG-infected mice

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Summary. The depressed antibody responses resulting from the administration of live BCG i.v. to mice have been investigated. The antibody response of spleen cells to SRBC or DNP-Ficoll *in vitro* was followed using Marbrook culture vessels. Depressed responses were also found *in vivo* confirming the results obtained *in vitro*. The response *in vitro* of normal spleen cells was suppressed by the addition of spleen cells from mice injected with BCG but not by the medium in which they had been growing for 2 days. The response of the normal spleen cells was also not suppressed by freeze/thaw disrupted BCG spleen cells, suggesting that the depressed responses in the mice injected with BCG are due to an active suppression by intact cells. This was confirmed by the cell-depletion experiments. Removal of cells from the BCG-primed cell populations using carbonyl iron or adherence to plastic not only abrogated the depressed responses but revealed an underlying enhancement of the immune response. The data suggest that the suppressive cell might be a macrophage.

INTRODUCTION

The study of depressed immune responses due to chronic infections with certain bacteria, viruses and protozoa (WHO, 1978) is important for two main reasons. Knowledge of the cause and possible prevention of the depression could be beneficial for the patient's progress. Secondly, the possibility of immunodepression resulting from administration of *Mycobacterium bovis*, BCG for immunoprophylaxis of leprosy or tuberculosis or for immunotherapy of cancer patients needs careful evaluation.

BCG administration can cause a depression of various immune responses (see for example Orbach-Arbouys & Poupon, 1978; Klimpel & Henney, 1978) including antibody production in mice against both T-dependent and T-independent antigens (Doft, Merchant, Johannessen, Chaparas & Sher, 1976). This depression is usually found after intravenous (i.v.) rather than subcutaneous (s.c.) injection of BCG and with relatively high doses. Enhancement, however, may alternatively occur depending on the exact conditions employed (Florentin, Huchet, Bruley-Rosset, Halle- Pannenko & Mathé, 1976; Doft *et al.*, 1976). The parameters determining whether enhancement or inhibition will occur are ill defined. Studying antibody formation *in vitro* by spleen cells from BCG-treated mice, Brown, Brown & Šljivić (1979) have shown that the viability of the BCG preparation is of great importance. The deliberate inclusion of 25% or more dead organisms in the inoculum resulted in an enhanced response to sheep red blood cells (SRBC) whereas the fully viable preparation injected i.v. caused depressed responses to both SRBC (T-dependent) and dinitrophenylated Ficoll (DNP-Ficoll; T-independent). The

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experiments reported here were designed to determine whether this depression was caused by a malfunctioning of one or more of the cell types involved in antibody production or alternatively was due to active suppression by cells or their products.

MATERIALS AND METHODS

Mice

Female CBA/Ca strain mice were bred in our animal house and used when 3 months old.

BCG

The Glaxo strain of BCG was used within 6 h of harvest from a 14-day culture in Glaxo glycerol-free medium or from Middlebrook 7H9 broth culture (Difco). The opacity was measured on a Spekker absorptiometer type H760 (Hilger and Watts Ltd, London) and the concentration of BCG organisms was then estimated from a graph prepared previously showing the opacity of various batches against their ability to form colonies on Middlebrook 7H10 agar plates (Difco) incubated at 37° for 21–28 days. This was found the most convenient way to estimate the number of organisms in a BCG preparation for use on the same day as harvest. Retrospective viable counts were determined as above on occasional preparations as a check.

Before use, a BCG preparation was diluted if necessary in growth medium and injected via a lateral tail vein to give a final dose of 10^8 organisms per mouse.

Antibody response in vitro

On the appropriate days after BCG injection, spleens were removed aseptically from injected and untreated mice. Spleens from at least two mice in each experimental group were pooled. Cell suspensions were prepared by washing and pushing the spleens through stainless steel sieves (mesh 80/inch). The medium used throughout was RPMI 1640 with added L-glutamine (Flow Laboratories, Irvine, Scotland) supplemented with 0·024 M sodium bicarbonate, 0·0147 M HEPES, 5% foetal calf serum (Flow Laboratories) and 10 units/ml gentamicin. The spleen cell suspensions were allowed to stand at room temperature for 5 min and then decanted from any cell clumps. The concentration was adjusted to 2×10^7 nucleated cells/ml. Cells were cultured in modified Marbrook chambers (Marbrook, 1967) which were constructed by suspending an inner glass tube (14 mm diameter), the lower end of which was closed with dialysis membrane, in a 30 ml plastic universal container (Sterilin Ltd, Teddington, Middlesex). One millilitre of the cell suspension was added to the inner tube together with 0·1 ml medium containing either 2×10^6 SRBC or 20 ng DNP-Ficoll. Medium alone (15–18 ml) was added to the outer chamber so that fluid levels in both chambers were the same. Quadruplicate cultures were used for each experimental group. The cultures were incubated for 4 days at 37° in a humidified incubator gassed to 5% CO_2. After harvest, the number of cells forming antibodies (AFC) against SRBC or trinitrophenylated SRBC (TNP-SRBC) (Rittenberg & Pratt, 1969) was determined using the method of Cunningham & Szenberg (1968).

Disrupted cells

Spleen cell suspensions were prepared from untreated and BCG-injected mice as above but adjusted to 4×10^7 cells/ml. Some of these cells were then frozen for 2 min in an acetone/dry ice freezing mixture and thawed for 5 min in a water bath set at 25°. This freezing and thawing was repeated four times, with the cells being well mixed on an electric mixer (Vortex-Genie) after each thawing.

Supernatants

Spleen cell suspensions prepared from untreated or BCG-injected mice were adjusted to 2×10^7 cells/ml and dispensed in volumes of 3 ml into 40 ml plastic tissue culture flasks (Nunc, Denmark). The flasks were incubated for 48 h at 37° in a humidified atmosphere containing 5% CO_2. The medium and floating cells were then pipetted from the flasks and centrifuged at 500 ***g*** for 10 min to remove cells and cell debris. Fresh medium (1·2 ml) was added to the cells remaining attached to the plastic floors of each of two tissue culture flasks used for BCG-primed cells after the supernatants had been removed. The cells were washed and scraped into the medium. The supernatants and collected cells were tested on the day of harvest. Their activity was assayed by adding them to normal spleen cells in Marbrook culture vessels as above.

Antibody response in vivo

Mice were injected i.v. into the tail vein with 10^7 SRBC at the appropriate time after BCG injection. The spleens were removed 4 days later and cell suspensions were prepared as above from each spleen individually. The suspensions were centrifuged at 700 ***g*** for 10 min

and the cells resuspended in 10 ml of medium. The cells were then counted and the number of AFC against SRBC was determined as above.

Removal of phagocytic cells

Triple-washed sterile carbonyl iron (powder type SF, GAF Ltd, Tilson Road, Manchester) was added to spleen cell suspensions (20 mg/2 × 10^7 cells/ml) and the mixture was incubated on a roller at 37° for 40 min. The carbonyl iron and associated cells were then removed by applying a strong magnet to the side of the container and tipping off the remainder of the cells. If necessary the magnet was applied a second time. The cells remaining in suspension after this procedure will be referred to as 'non-phagocytic'; no attempt was made to adjust the cell concentration to that of the unfractionated cells.

Removal of adherent cells

Spleen cell suspensions containing 2 × 10^7 cells/ml were pipetted into 90 mm Sterilin (Sterilin Ltd, Teddington, Middlesex) petri dishes (approximately 5 ml/dish) and incubated in a CO_2 incubator at 37° for 30 min. Using a pasteur pipette, the cells were washed fairly vigorously over the surface of the dish so that only the strongly adherent cells would remain on the plastic. The unattached cells were then transferred to a second set of petri dishes and the incubation and washing repeated. The cells remaining unattached after this procedure will be referred to as 'non-adherent'. As above, no attempt was made to adjust the cell concentration to that of the unfractionated cells.

RESULTS

Unfractionated cells and their mixtures

In accordance with the earlier report (Brown *et al.*, 1979), spleen cells from mice injected i.v. 10 or 13 days previously with 10^8 live BCG gave a depressed response to SRBC *in vitro* compared to that of cells from untreated mice (Fig. 1). The addition of 2 × 10^7 cells from BCG-injected mice to the same number of cells from untreated animals also resulted in a low response only slightly higher than that of the BCG-primed cells alone. Although the response of the normal cells was also lowered by the addition of a further 2 × 10^7 normal cells, this suppression was much less than that caused by the addition of the same number of cells from BCG-treated mice.

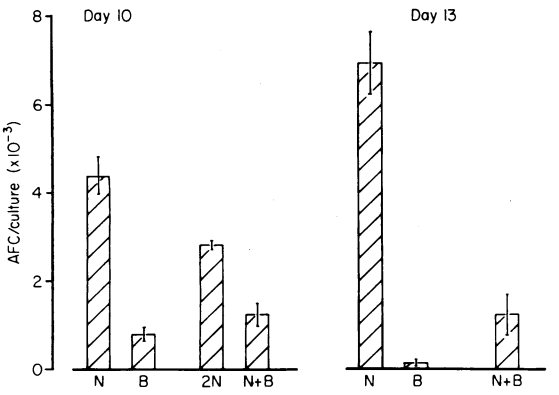

Figure 1. Effect of mixing spleen cells from untreated (N) and BCG-injected (B) mice on their antibody response *in vitro* to SRBC. Mice were injected i.v. with 10^8 live BCG 10 or 13 days before removal of the spleens.

Disrupted BCG-primed spleen cells

The finding that cells from mice injected with live BCG suppressed the antibody response of cells from untreated mice suggested that this depression was an active process rather than a malfunctioning of a cell type. To see whether intact cells were necessary for this suppressive activity, cells from BCG-injected mice were disrupted by repeated freezing and thawing. Their activity was then tested on spleen cells from untreated mice. The undisrupted spleen cells from the mice injected with BCG gave fewer AFC than spleen cells from untreated mice as expected and when mixed with the latter were capable of suppressing their response (Table 1). In contrast, the disrupted cells did not show this suppressive activity.

Table 1. Effect of freeze–thawing on the depression by cells from BCG-injected mice

Cells in culture (millions)			
	BCG-injected donors*		
Untreated donors	Untreated	Freeze–thawed	AFC/culture
20	—	—	7150 ± 2024
—	20	—	3415 ± 1213
20	20	—	3165 ± 740‡
20	—	20	5820 ± 552
20	—	Filtrate†	7560 ± 492

* Thirteen days after injection.
† Filtrate of 20 × 10^6 cells.
‡ Significantly different ($P < 0.05$) from cells from untreated donors.

Supernatants from BCG-primed spleen cells

If freeze/thaw disrupted cells from mice injected with live BCG were unable to cause suppression, it seemed likely that the suppression was due to the action of the intact cells themselves or due to a factor released from them into the medium and capable of affecting the response of other cells. Spleen cells from BCG-injected mice were incubated for 48 h before the medium was collected and centrifuged to remove floating cells. This medium was then tested for suppressive activity by incubating it with spleen cells from untreated mice in Marbrook cultures for the usual 4 days. As can be seen from Table 2, supernatants from incubated spleen cells from untreated mice had no effect on the AFC response to SRBC of further normal spleen cells and supernatants from BCG-primed spleen cells tended to enhance rather than suppress the response of the normal cells. To ensure that the spleen cells used to produce the supernatant were capable of producing suppression, some of the cells remaining attached to the tissue culture flask after removal of the supernatant were also incubated with the normal cells and did produce the expected suppression.

Table 2. Effect of spleen cell culture supernatants on the antibody response of normal spleen cells

Additions to cultures*	AFC/culture
None	7230 ± 311
0·5 ml supernatant (BCG)	10595 ± 1028†
0·1 ml supernatant (BCG)	8710 ± 899
0·5 ml supernatant (normal)	7125 ± 1018
0·1 ml supernatant (normal)	7435 ± 695
0·5 ml adherent cells (BCG)	2620 ± 309‡

*Spleen cells from untreated mice or mice injected 11 days previously with live BCG were cultured for 2 days. Cell-free supernatants or adherent cells from these cultures were added to normal cells.

Significance of differences from 'none' control cultures †$P < 0.05$; ‡$P < 0.001$.

Antibody response *in vivo*

The depressed responses to SRBC and DNP-Ficoll *in vitro* could have been an artefact due, for example, to an imbalance of cell types in the culture chambers. To investigate whether the depression was a genuine result of the treatment with BCG and not due to artificial culture conditions, the antibody response *in vivo* was assessed at a time after BCG injection when the response *in vitro* would have been depressed maximally. The results are shown in Table 3. The number of AFC to SRBC *in vivo* was depressed significantly whether the results were expressed as AFC/10^6 cells or as AFC/spleen to allow for the increase in spleen size in the BCG-treated mice.

Table 3. Antibody response *in vivo* of mice injected with live BCG

Group	AFC/spleen	AFC/10^6 spleen cells
Control	5·89 ± 0·13 (768,158)	3·67 ± 0·09 (4,686)
BCG-injected	5·28 ± 0·11* (190,856)	2·56 ± 0·07† (365)

Control and injected (10 days after BCG) mice were immunized i.v. with 10^7 SRBC and the number of AFC determined 4 days later. AFC numbers are given as mean $\log_{10} \pm$ SEM and geometric means (in parentheses) for three mice in each group.

* $P < 0.025$.

† $P < 0.001$.

Repeated changes of medium

As a further check that the depressed responses by cells from BCG-injected mice were not due to depletion of nutrients or important factors from the medium, the medium in the outer chambers of the Marbrook culture vessels was changed daily throughout the 4 day incubation period. As shown in Table 4, this procedure increased slightly the number of AFC in cultures of spleen cells from both untreated and BCG-injected

Table 4. Effect of daily changes of culture medium on the antibody response *in vitro* of spleen cells from untreated or BCG-injected mice

	AFC/culture	
Number of medium changes	Untreated mice	BCG-injected mice*
0	4125 ± 504	975 ± 224
3	4910 ± 207	1625 ± 230

* Spleen cells prepared 12 days after injection of 10^8 BCG organisms.

Table 5. Effect of removal of plastic-adherent or phagocytic cells on the antibody response *in vitro* of spleen cells from untreated (N) or BCG-injected (BCG) mice

Days after injection*	Cells in culture			AFC/culture		Cell recovery†
	Unfractionated	Non-adherent	Non-phagocytic	Anti-SRBC	Anti-DNP	
10	N	—	—	6,425 ± 904		
	—	—	N	660 ± 385		67
	BCG	—	—	455 ± 36		
	—	—	BCG	13,750 ± 5839		39
	N + BCG	—	—	1,360 ± 250		
14	BCG	—	—	1,705 ± 392		
	—	—	BCG	7,230 ± 530		47
	—	BCG	—	3,565 ± 192		53
20	N	—	—	3,465 ± 473		
	—	N	—	2,350 ± 307		59
	BCG	—	—	2,985 ± 500		
	—	BCG	—	5,380 ± 379		76
13	N	—	—	8,625 ± 1536		
	—	—	N	2,887 ± 327		57
	BCG	—	—	2,495 ± 744		
	—	—	BCG	25,115 ± 1403		76
14	N	—	—	2,835 ± 373	3430 ± 181	
	—	N	—	1,230 ± 208	3180 ± 144	
	BCG	—	—	2,650 ± 219	3675 ± 516	
	—	BCG	—	6,780 ± 249	6665 ± 694	

* A separate batch of BCG was used for each day shown.
† Percentage of cells recovered after depletion procedures. No adjustment of cell concentration was made.

mice, but the difference between the two groups remained. Thus with unchanged medium in the Marbrook vessels the number of AFC from the BCG-treated mice was 24% that from untreated mice and with fresh medium daily the corresponding figure was 33%.

Addition of 2-mercaptoethanol to the cells at the beginning of the incubation period had a similar effect (results not given); spleens from untreated mice and mice injected with live BCG both had a slightly increased number of AFC to SRBC *in vitro* in the presence of 2-mercaptoethanol but the cells from the BCG mice still showed pronounced depression.

Removal of adherent or phagocytic cells

The effect of removing adherent or phagocytic cells from the spleen suspensions before adding them to the Marbrook vessels is shown in Table 5. The depleted cell populations were added in a volume of 1 ml; the percentage of the original cell number this represented is indicated in the last column. The number of cells removed by adherence to plastic or by phagocytosis of carbonyl iron varied considerably. There was no consistent difference between the number removed by either process or between cell suspensions from BCG-treated mice rather than untreated mice. In all experiments, removal of phagocytic or adherent cells from spleen preparations from untreated mice decreased the number of AFC against SRBC. The last experiment shows that removal of adherent cells from a spleen suspension from untreated mice had no effect on the number of AFC against DNP-Ficoll. In contrast, removal of the phagocytic or adherent cells from spleen cell preparations from mice injected with live BCG resulted in numbers of AFC against SRBC or DNP-Ficoll which were higher than the values for the unfractionated cells from both BCG-injected and untreated mice. The last experiment and that performed 20 days after BCG injection also show that suppressing cells can be demonstrated even at a stage in the infection when the response of the cells appears normal.

DISCUSSION

We have reported elsewhere that spleen cells taken from mice injected i.v. 10–15 days previously with 10^8 viable BCG produce fewer AFC *in vitro* against SRBC and DNP-Ficoll than cells from untreated mice (Brown *et al.*, 1979). It was postulated that this depression could be due to the presence of suppressor cells or alternatively, since antibody production requires the co-operation of more than one cell type, to the failure of one or more of these types to function efficiently. Since the response to DNP-Ficoll is T-cell independent it was thought unlikely that the depression was the result of malfunctioning T helper cells. The macrophages in the spleen of BCG-injected mice are known to be carrying a burden of metabolising, and possibly multiplying, organisms. The antibody response to DNP-Ficoll is however relatively macrophage independent both *in vivo* (Ishizaka, Otani & Morisava, 1977) and *in vitro* (Rumjanek, Watson & Šljivić, 1977; Table 5) and lack of macrophage co-operation is unlikely to be the sole cause of the low responses of the spleen cells from the BCG-treated mice. Some direct effect on B cells was possible.

The results presented here show that an active suppressive process is occurring 10–15 days after injection with live BCG and that it is actually masking an enhanced response to SRBC. The suppressive activity could not be detected in freeze/thaw disrupted spleen cells. Neither could it be found in the cell-free medium surrounding BCG-primed cells after they had been incubated for 2 days even though the adherent cells from these cultures were themselves suppressive. This would suggest that an active process by living cells must be involved (but see below for further discussion on cell factors). All results from techniques using conditions *in vitro* need to be evaluated carefully. BCG is known to stimulate macrophages and it is possible that the medium in the BCG-primed cell populations was becoming exhausted after 4 days incubation and unable to maintain lymphocyte activity. However, since daily changes of medium in the outer containers of the Marbrook culture vessels did not prevent the depression, this is unlikely to be of major importance. Depressed responses to SRBC were also found *in vivo* after BCG injection confirming the results found in the experiments *in vitro*.

No suppressive activity could be detected in the medium taken from a 48 h culture of spleen cells from mice injected with BCG 11 days previously, even when used at a concentration of one part medium to two parts of normal cell suspension. However, since the supernatant was added only to the inner tube of the Marbrook chamber, any active low molecular weight material could have passed through the dialysis membrane and been diluted considerably in the medium in the outer chamber. An inhibitory factor of low molecular weight has been described by Calderon & Unanue (1975). Furthermore, it is known that spleen cells taken at later stages after BCG injection produce stimulatory factors even when they themselves do not show enhanced responses (Brown, Brown & Šljivić, 1978). These factors are not diluted out in the Marbrook system. It is possible that any inhibitory activity was counterbalanced by these stimulating factors. It should be remembered, however, that any low molecular weight factors produced by whole cells added to the Marbrook chambers would also be dialysed, and repeated changes of medium had little effect on the depressed response.

Removal of cells which were phagocytic or adherent to plastic removed the suppressive activity of the BCG spleen preparations suggesting that the suppressive cell might be a macrophage. Two considerations should however be discussed here. First, the depressed responses in the unfractionated cell suspensions from the BCG-treated mice could be due to an excess of cells with phagocytic and adherent properties. Excess macrophages are known to be suppressive (Baird & Kaplan, 1977) and this may be the explanation for the lower response of 4×10^7 normal cells than of 2×10^7 cells per culture. If this is the case, it is unlikely to be due solely to artificial conditions *in vitro* because depressed responses were also found *in vivo* after i.v. injection with 10^8 live BCG. Secondly, the possibility of loss of other cell types which become attached to macrophages and could be removed with them cannot be excluded. This is supported by the present observation of high loss of cells after depletion procedures.

Although most reports agree that depression of lymphocyte responses in other systems caused by pretreatment with BCG depends on i.v. rather than s.c. administration and a high dose of organisms, there is disagreement on the nature of the suppressor cell. Orbach-Arbouys & Poupon (1978) found that T-enriched populations from spleens of BCG-treated mice, as well as adherent cells, lowered the PHA reactivity of normal T cells. The same cell populations also inhibited the growth of tumour cells *in vitro*. Doft *et al.* (1976) suggest a suppressor macrophage by a process of elimination whereas Sultzer (1978) favours a suppressor T cell. Klimpel & Henney (1978) conclude that

a macrophage-like spleen cell from mice given BCG i.p. is the cause of suppressed proliferative and cytotoxic responses of normal lymphocytes. Some of these contradictions can perhaps be explained by the paper by Huchet & Florentin (1976) on the nature of the suppressor cell. They found suppressive adherent cells when 1 mg BCG was given to mice but at higher doses they found suppressive T cells in addition. Bennett, Rao & Mitchell (1978) suggest the nylon-wool-adherent suppressor cells found by them in the spleen after BCG treatment originate in the bone marrow and are activated natural suppressor cells.

In conclusion, we suggest that depressed AFC responsiveness to both SRBC and DNP-Ficoll is an active process and not due to malfunctioning cells. The suppressing cells mask an underlying enhancement of antibody responses. The cells responsible for the suppression are removed with adherent and phagocytic cells and are likely to be macrophages. The possibility of simultaneous removal of other cells has not yet been excluded. The fact that responses to SRBC of cells from untreated mice were only reduced and not abolished by these procedures suggests that (1) only the more strongly phagocytic or adherent cells were removed and/or (2) immature cells were becoming functional during the 4 day incubation period in the Marbrook vessels. If the first proposition is true, the cells with weaker properties cannot be suppressive and similarly if the second proposition is true, these recently matured macrophages cannot be suppressive. A heterogeneity of the macrophage population seems likely in either case.

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REFERENCES

BAIRD L.G. & KAPLAN A.M. (1977) Macrophage regulation of mitogen-induced blastogenesis. II. Mechanism of inhibition. *Cell. Immunol.* **28,** 36.

BENNETT J.A., RAO V.S. & MITCHELL M.S. (1978) Systemic bacillus Calmette-Guérin (BCG) activates natural suppressor cells. *Proc. natn. Acad. Sci. (U.S.A.)*, **75,** 5142.

BROWN C.A., BROWN I.N. & ŠLJIVIĆ V.S. (1978) Enhancement of the antibody response *in vitro* by BCG. *Dev. biol. Stand.* **38,** 153.

BROWN C.A., BROWN I.N. & ŠLJIVIĆ V.S. (1979) Suppressed or enhanced antibody responses *in vitro* after BCG treatment of mice: importance of BCG viability. *Immunology*, **38,** 481.

CALDERON J. & UNANUE E.R. (1975) Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. *Nature (Lond.)*, **253,** 359.

CUNNINGHAM, A.J. & SZENBERG A. (1968) Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology*, **14,** 599.

DOFT B.H., MERCHANT B., JOHANNESSEN L., CHAPARAS S.D. & SHER N.A. (1976) Contrasting effects of BCG on spleen and lymph node antibody responses in nude and normal mice. *J. Immunol.* **117,** 1638.

FLORENTIN I., HUCHET R., BRULEY-ROSSET M., HALLE-PANNENKO O. & MATHÉ G. (1976) Studies on the mechanisms of action of BCG. *Cancer Immunol. Immunother.* **1,** 31.

HUCHET R. & FLORENTIN I. (1976) Studies of mechanism of action of BCG. In: *BCG in Cancer Immunotherapy* (Ed. by G. Lamoureux, R. Turcotte and V. Portelance), p. 85. Grune & Stratton, New York.

ISHIZAKA S., OTANI S. & MORISAVA S. (1977) Effect of carrageenan on immune responses. I. Studies on the macrophage dependency of various antigens after treatment with carrageenan. *J. Immunol.* **118,** 1213.

KLIMPEL G.R. & HENNEY C.S. (1978) BCG-induced suppressor cells. I. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T-cell generation *in vitro*. *J. Immunol.* **120,** 563.

MARBROOK J. (1967) Primary immune response in cultures of spleen cells *Lancet*, **ii,** 1279.

ORBACH-ARBOUYS S. & POUPON M.-F. (1978) Active suppression of *in vitro* reactivity of spleen cells after BCG treatment. *Immunology*, **34,** 431.

RITTENBERG M.B. & PRATT K.L. (1969) Anti-trinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen. *Proc. Soc. exp. Biol. Med.* **132,** 575.

RUMJANEK V.M., WATSON S.R. & ŠLJIVIĆ V.S. (1977) A re-evaluation of the role of macrophages in carrageenan-induced immunosuppression. *Immunology*, **33,** 423.

SULTZER B.M. (1978) Infection with Bacillus Calmette-Guérin activates murine thymus-independent (B) lymphocytes. *J. Immunol.* **120,** 254.

WORLD HEALTH ORGANISATION (1978) Immunodeficiency. *Tech. Rep. Series*, No. 630, W.H.O., Geneva.