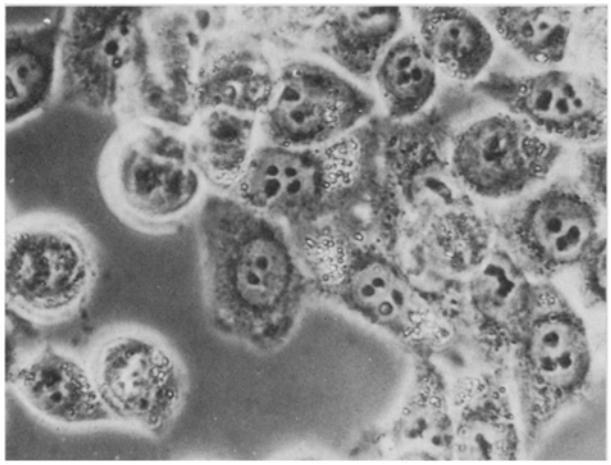

Fig. 3. Group of living HeLa cells immediately before treatment with toad serum. Phase contrast.

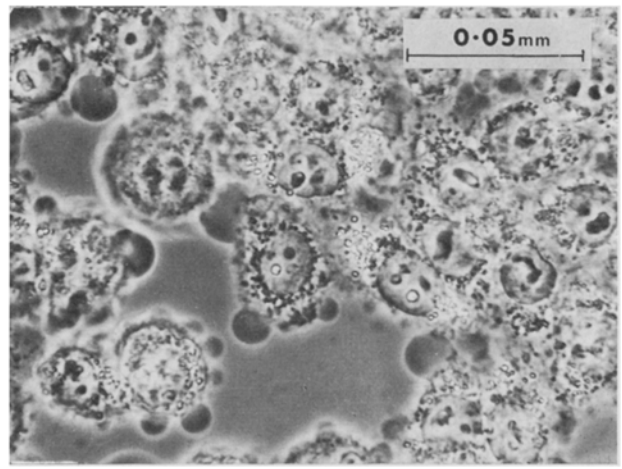

Fig. 4. Same group of HeLa cells as in Figure 3, photographed 20 min after addition of 50% toad serum to the medium. Phase contrast.

guinea-pig; its action against erythrocytes is selectively destructive[1].

Our results suggest that the destruction of mammalian tumour transplants in toads may have been due primarily to a naturally occurring, heat-labile, cytotoxic factor or factors in body fluids. They do not exclude the possibility of a concurrent, cell-mediated response in unconditioned toads. *Bufo marinus* can produce antibodies over a wide range of temperatures[5] including 37 °C[6].

Our results add to the available phylogenetic information regarding the natural toxicity of serum of specific animals for specific cell types. The role of the cytotoxicity of natural sera in the defence mechanisms of host animals will become apparent only as a greater range of data, particularly from in vitro studies, becomes available[7].

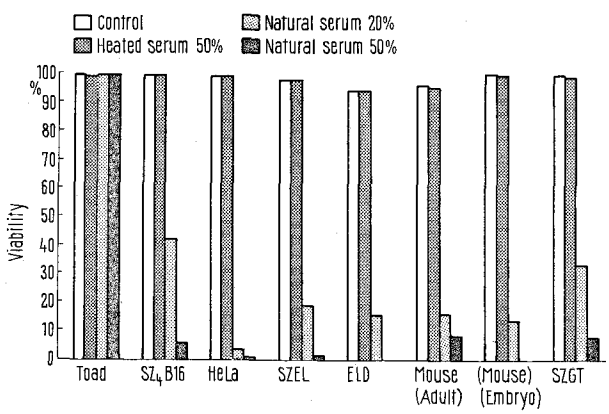

Fig. 5. Histogram showing percentage viability of dissociated normal and malignant cells exposed to toad serum for 30 min at 37°C. In all except mouse and SZGT, figures are based on means of at least 3 experiments with different batches of pooled serum.

Résumé. Le sérum et le plasma, nouvellement préparés, de l'Amphibien *Bufo marinus*, ont, in vitro, un effet rapidement cytotoxique sur des cellules normales et malignes des autres Vertébrés.

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[5] N. N. Tait, Physiol. Zool. *42*, 29 (1969).
[6] E. Diener and J. Marchalonis, Immunology *18*, 279 (1970).
[7] This work was supported by a grant from the University of Sydney Cancer Research Fund. We thank Miss Cynthia Batson for technical assistance.

Specific Stimulation of Mycobacteria Phagocytosis by Substances Liberated during the Cultivation of Lymph Node Cells from Tuberculin Hypersensitive Rabbits with the Specific Antigen

Macrophages play the crucial role in immunity against intracellular parasites[1]. They act, however, rather as pharmacologically active effector cells, whereas the proper immunologically specific process is triggered by the lymphocytes[2,3].

In a previous paper we have demonstrated the role of immune lymphocytes in the phagocytosis of foreign erythrocytes by peritoneal macrophages from normal non-sensitized animals[4]. In the present paper the stimulation of phagocytosis by mediators of delayed hypersensitivity[5] liberated during the interaction of hypersensitive lymphocytes with the specific antigen was studied.

For this purpose, live Bacillus Calmette-Guérin (BCG) vaccine (in homogenized suspension) was added to cultures of normal peritoneal macrophages cultured in Parker 199 (GIBCO) without or in the presence of supernatants prepared by the cultivation of lymph node cells from tuberculin hypersensitive rabbits with various doses or

Table I. The effect of various biologically active supernatants on the phagocytic activity

Substance tested	Phagocytic activity after 2 h incubation	
	Extracellular *Mycobacteria* (%)	Macrophages with phagocytized *Mycobacteria* (%)
Tuberculin sensitive		
Normal cultivation medium	38–42	9.5
Supernatant A	31–38	10.0
Supernatant B	0– 1	20.0
Supernatant C	8–11	10.5
Supernatant C + 50 µg PPD	1– 2	18.5
Encephalitogen hypersensitive		
Supernatant A	25–40	10.5
Supernatant B	35–40	11.0

The values given represent an average calculated from 8 experiments. Supernatant: A, culture without antigen; B, culture with 100 µg PPD/ml; C, culture with 1 µg PPD/ml; C, + 50 µg PPD/ml added.

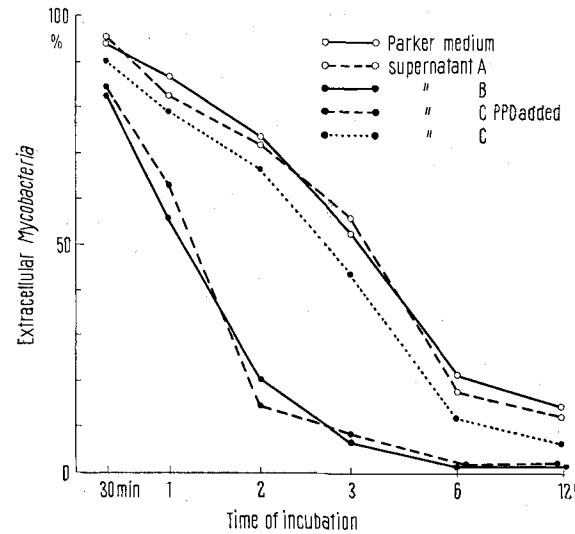

Fig. 1. The effect of various supernatants on the percentage of extracellular (nonphagocytized) *Mycobacteria* (BGC) in different incubation periods.

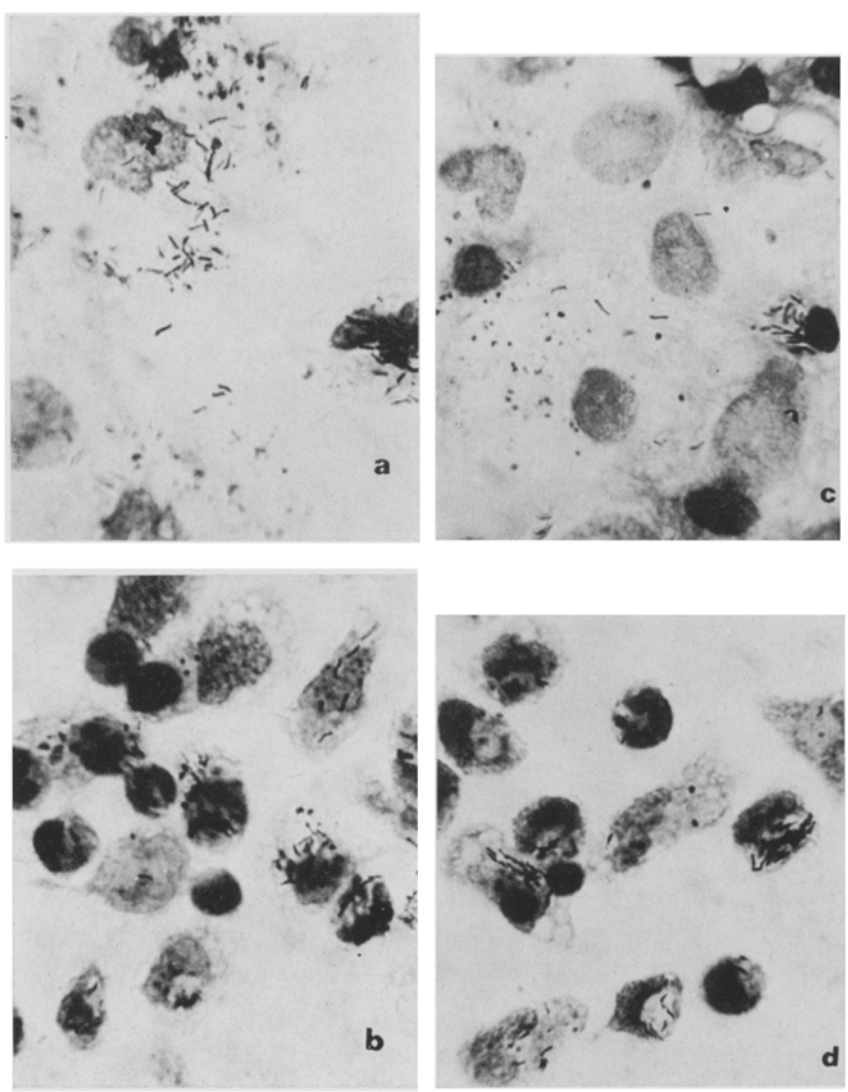

Fig. 2. The effect of various supernatants on the phagocytosis of *Mycobacteria* (BCG) with normal macrophages after 2 and 6 h incubation. Ziehl-Nielsen and Giemsa stain. Incubation time: a) supernatant A, 2 h; b) supernatant B, 2 h; c) Supernatant A, 6 h; d) Supernatant B, 6 h.

Table II. Effect of various biologically active supernatants on the phagocytosis of latex particles (DIFCO) and Staphylococci by normal macrophages

Substance hypersensitive to:	Phagocytic activity after 2 h incubation		
	Latex particles		Staphylococci
	Extracellular (%)	No. of cells with ingested particles (%)	No. of cells with ingested bacteria (%
Tuberculin			
Culture medium	35–42	12	14
Supernatant (A)	35–40	14	12
Supernatant (B)	38–42	11	12
Encephalitogen			
Supernatant (A)	35–40	11	11
Supernatant (B)	35–40	13	12
Sheep erythrocytes			
Supernatant (A)	35–40	12	12
Supernatant (B)	35–40	12	13

without the specific antigen[6]. The amount of intracellular and extracelluar *Mycobacteria* as well as the amount of phargocytic cells were then followed. For each individual value 200 cells and the corresponding number of fields in the microscope were examined. Evaluation of nonspecific aggregation of *Mycobacteria* to macrophages was excluded by careful observation of each phagocyting cell. In control experiments the suspension of *Staphylococcus pyogenes* and the latex particles (DIFCO 0.81) were used. The results are expressed in percent in all the indicators studied.

Principally two kinds of biologically active substances were studied for their phagocytosis stimulating effect. Supernatant designated 'A' was prepared by cultivation of lymph node cells without antigen and served as a negative control. Supernatant called 'B' containing the migration inhibitory factor (MIF) was prepared by cultivation with a high dose of the specific antigen[6] and the antigen dependent factor (supernatant 'C') was raised by the cultivation with a small amount of antigen[7,8]. Supernatant C was tested either alone or with addition of 50 μg PPD tuberculin[7,8]. Besides supernatants from tuberculin hypersensitive rabbits also preparations from purified encephalitogen or sheep red cells sensitized animals were used[9].

The macrophages phagocytized the *Mycobacteria* partially also in a normal culture medium or in the presence of a control supernatant A. An experimental arrangement was therefore elaborated enabling the evaluation of the phagocytose stimulating effect of the biologically active mediators, the phagocytosis was followed in several time intervals (Figure 1). Positive results were obtained using B and C + antigen supernatants. The effect was time-dependent; optimal differences from control experiments were found between 1–3 h of incubation. In earlier and later intervals they were less marked and after 12 h already negligible. The interval of 2 h was used in further experiments as the most suitable. A further problem was the quantitation of the phagocytic activity. As best criterion, the enumeration of the nonphagocyted *Mycobacteria* and the amount of macrophages with ingested microbes were used. After 2 h cultivation the cultures influenced by active supernatants were characterized by a pronounced phagocytic activity; many cells contained phagocytized *Mycobacteria* and outside the cells very few if any free *Mycobacteria* could be found (Figure 2). After 6 h of incubation there is a complete phagocytosis in active supernatant B and only slight signs of phagocytosis in control supernatant A.

Results, summarized in Table I, clearly show a marked stimulation of the phagocytic process, which is apparent especially in cultures influenced by the supernatants B and C with the addition of 50 μg PPD.

In control experiments we were unable to find a nonspecific potentiation of the phagocytic activity of latex particles of Staphylococci (Table II). The amount of ingested particles or bacteria and the amount of phagocytic cells were identical in all groups. Also unrelated, delayed hypersentivity systems yielded negative results (Tables I and II).

The results point to the view that the process of *Mycobacteria* phagocytosis was markedly influenced by biologically active substances liberated during the interaction of hypersensitive lymph node cells with the specific antigen. The phagocytosis process was accelerated and intensified in the presence of the above-mentioned substances. This process was immunologically specific, similarly as found in our previous paper dealing with the phagocytosis of sheep and rooster erythrocytes[4].

Important is the finding with the antibody-like factor contained in the supernatant C. This factor has the capacity to bind to normal nonsensitized macrophages and to influence their behaviour so that they can react specifically with the respective antigen by inhibition of

[1] G. B. Mackaness and R. V. Blanden, Progr. Allergy *11*, 89 (1967).
[2] G. B. Mackaness and W. C. Hill, J. exp. Med. *129*, 993 (1969).
[3] WHO, Technical Reports Series *423* (1969).
[4] K. Barnet, J. Pekárek and J. Johanovský, Experientia *24*, 948 (1968).
[5] H. S. Lawrence and M. Landy, *Mediators of Cellular Immunity* (Academic Press, New York 1969).
[6] J. Švejcar, J. Pekárek and J. Johanovský, Immunology *15*, 13 (1968).
[7] J. Švejcar, J. Pekárek and J. Johanovský, Folia microbiol., Praha *13*, 247 (1968).
[8] J. Švejcar, J. Pekárek, J. Johanovský, Z. Immun Forsch. exp. Ther. *138*, 283 (1969).
[9] J. Švejcar, J. Pekárek and J. Johanovský, to be published.

migration[10] or by specific stimulation of phagocytosis. The exact nature of this respective substance is not yet clear but apparently it is not a known immunoglobulin. It is known, however, that this type of substance is present also in supernatant B with distinct migration inhibitory activity[10]. The significance of this substance for the intracellular digestion process of *Mycobacteria* (the proper Mackaness-type of immunity) will be dealt with in another paper.

Zusammenfassung. Es wurde festgestellt, dass die Mediatoren der Überempfindlichkeit des verzögerten Types die Fähigkeit besitzen, die Phagocytose der Mykobakterien durch normale Makrophagen zu unterstützen. Die Wirkung hat sich als immunologisch spezifisch bewiesen.

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[10] J. ŠVEJCAR, J. PEKÁREK and J. JOHANOVSKÝ, Z. Immun Forsch. exp. Ther. *138*, 342 (1969).

A Stimulant Effect of Anti-Macrophage Serum on Antibody Production

During the last decade, evidence has suggested the important role of macrophages in antibody synthesis[1,2]. Recently this essential role of phagocytic cells seems, however, to be somewhat weakened. It was reported that these cells inhibited rather than enhanced the immune response[3–6]. Very recently clear evidence has been presented that both induction of immunity and tolerance occured in vitro in the absence of macrophage[7]. A new possibility to study this problem is the administration of anti-macrophage serum (AMS). Some investigators have found no immunosuppression in mice treated with AMS[8–10]. Others reported impairment of antibody production only under limited conditions[11,12].

In this report we present preliminary data of the enhancing effects of AMS on antibody synthesis of mice immunized with a single large dose of sheep RBC.

AMS was produced in rabbits with peritoneal cells from Swiss donor mice. The lymphocytes were not eliminated from the peritoneal exudate. Rabbits were inoculated twice at 3-week intervals, s.c. with 10^8 cells in complete Freund adjuvant. The animals were bled 1 week after the last injection. The serum was absorbed with spleen cells. Data of agglutinating antibodies in AMS before and after the absorption with spleen cells are presented in Table I. Cytotoxicity of AMS was tested in vitro and in vivo[13,14]. In vitro cytotoxicity titre to macrophages was 1:3670. The in vivo administration of 0.5 ml AMS caused marked change in the cellular content of the peritoneal exudate: the number of viable macrophages was reduced to 7% as compared to control mice treated with normal rabbit serum (NRS).

Table I. Titre of agglutinating antibodies in AMS

	Titre to		
	Macrophages	Lymphocytes	Erythrocytes
AMS	1/2187	1/135	1/5
AMS absorbed with spleen cells	1/729	1/5	0

Effect of AMS on antibody synthesis. Swiss mice weighing 25–30 g were pretreated with 0.5 ml AMS i.p. and 24 h later were immunized with 0.5×10^9 sheep RBC, i.p. AMS treatment was repeated on the 4th day; experiments were made on the 5th day. Antibody formation was assessed by a modification of the Jerne haemolytic plaque technique[15] using agarose and glass microscope slides[16] and the values were expressed as the number of haemolytic plaques/10^6 recovered cells. Each group consisted of 10 mice and average values of 7 parallel determinations were

Table II. Number of haemolytic plaques/per 10^6 spleen cells in mice immunized with 0.5×10^9 sheep RBC

Experiment	Treatment			
	AMS		Saline	
	Mean	±SE	Mean	±SE
1	77.4	13.3	53.6	27.8
2	264.3	68.3	75.2	20.1
3	295.0	69.1	275.0	48.6
4	143.8	25.1	182.8	61.6
5	239.8	34.7	94.6	9.5

AMS was administered 1 day before and 2 days after the immunization. Experiments were made 1 day after the 2nd AMS treatment. Control groups Were administered saline.

[1] M. FISHMAN and F. L. ADLER, J. exp. Med. *117*, 595 (1963).
[2] B. A. ASKONAS and J. M. RHODES, Nature, Lond. *205*, 470 (1965).
[3] G. HARRIS, Immunology *9*, 529 (1965).
[4] E. H. PERKINS and T. MAKINODAN, J. Immun. *94*, 765 (1965).
[5] R. E. M. PARKHOUSE and R. W. DUTTON, J. Immun. *97*, 663 (1966).
[6] R. D. MOORE and M. D. SCHOENBERG, Nature, Lond. *219*, 297 (1968).
[7] E. DIENER, K. SHORTMAN and P. RUSSEL, Nature, Lond. *225*, 731 (1970).
[8] E. R. UNANUE, Nature, Lond. *218*, 36 (1968).
[9] G. LOEWI, A. TEMPLE, A. P. P. NIND and M. AXELRAD, Immunology *16*, 99 (1969).
[10] J. P. DESPONT and A. CRUCHAUD, Nature, Lond. *223*, 838 (1969).
[11] J. F. JENNINGS and L. A. HUGHES, Nature, Lond. *221*, 79 (1969).
[12] B. F. ARGYRIS and D. H. PLOTKIN, J. Immun. *103*, 372 (1969).
[13] A. THIERFELDER, ED. MÖLLER and I. KIMURA, Z. ges. exp. Med. *147*, 44 (1968).
[14] E. THORSBY and F. KISSMEYER-NIELSEN, Vox Sang. *14*, 417 (1968).
[15] N. K. JERNE, A. A. NORDIN and C. HENRY, in *Cell Bound Antibodies* (Wistar Institute Press, Philadelphia 1963), p. 109.
[16] J. STERZL and L. MANDEL, Folia microbiol., Praha *9*, 173 (1964)