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RELEASE OF INTERFERON BY MOUSE PERITONEAL CELLS IN VITRO. THE REQUIREMENT OF CONTACT WITH ENDOTOXIN AND THE TEMPERATURE DEPENDENCE OF RELEASE

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Summary. — The optimal conditions for interferon release by mouse peritoneal leukocytes in vitro after stimulation with endotoxin required a contact time of about 20 minutes between the stimulator and the cells. Thereafter the endotoxin could be removed from the medium without impairing the subsequent interferon release. The temperature of 26° C, which proved to be optimal for interferon release in this system, was required only for the first 4 hours after addition of endotoxin. This phase was located temporally before that of the main interferon release ("temperature-sensitive stimulatory phase"). The possible implications of these findings to the loss of the ability to release interferon stimulated by endotoxin in vitro by mouse peritoneal cells are discussed.

Introduction

The intravenous application of bacterial endotoxins leads to appearance of circulating interferons in sera of experimental animals (Ho, 1964; Stinebring and Youngner, 1964; and others). The authors who tried to reproduce this endotoxin effect under in vitro conditions succeeded as yet only in 3 cases. Kono (1967) reported that an interferon-like inhibitor could be demonstrated in bovine leukocyte cultures treated with endotoxin. Recently, Kobayashi *et al.* (1968 — personal communication via ISM) found mouse spleen cells explanted in vitro to produce interferons after stimulation with endotoxin. Lackovič *et al.* (1967) observed that explanted mouse peritoneal cells release interferon regularly after stimulation with endotoxic extracts from various *Escherichia coli* strains. In their experiments the release of interferon showed a temperature optimum at 26° C, whereas the virus-induced interferon production by these cells was optimal at about 39° C (Lackovič *et al.*, 1967).

We investigated the time requirement for contact between peritoneal cells and an endotoxin obtained from *E. coli* 0111 : B4 necessary for the

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stimulation of interferon release in vitro, as well as the temporal relations between the appearance of interferon in the culture and the necessity of incubation of cells at 26° C for this event.

Materials and Methods

The preparation and cultivation of mouse peritoneal cells, the preparation of the endotoxic extract from *E. coli* 0111 : B4 (endotoxin), and the method used in interferon assay in mouse fibroblasts (L cells) were described previously (Borecký and Lackovič, 1964; Lackovič and Borecký, 1965; Lackovič *et al.*, 1967). In all experiments, the same preparation (*E. coli* 0111 : B4) of endotoxin was used. The interferon titres were expressed as reciprocals of the highest dilution of the interferon material which completely protected the L cells from the cytopathic effect of encephalomyocarditis (EMC) virus.

Results

The duration of contact between peritoneal cells and endotoxin as a requirement for interferon stimulation in vitro

Peritoneal cells were treated with endotoxin at 26° C immediately after establishing the cell cultures in tubes. Medium 199 with 10% foetal calf serum containing the endotoxin was then removed at intervals and replaced by a fresh medium of the same composition without endotoxin and the cultures were further incubated at 26° C. The interferon titres were determined in each sample after 24 hours and compared with controls incubated in the presence of endotoxin for the whole period of 24 hours. In other tubes the medium was changed for a new one containing the same amount of endotoxin as originally present. The interferon titres determined in such

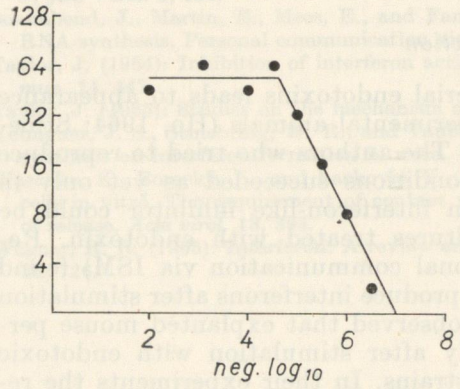

Fig. 1.

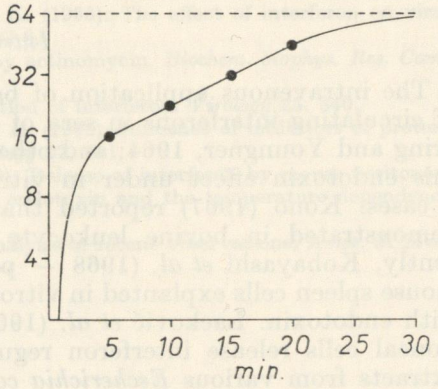

Fig. 2.

Fig. 1

The yield of interferon in mouse peritoneal cells treated with various amounts of endotoxin at 26° C in vitro

Abscissa: dilution of endotoxin; ordinate: interferon titre per ml after 24 hours

Fig. 2.

The yield of interferon in mouse peritoneal cells kept in contact with endotoxin at 26° C for various periods of time

Abscissa: time of contact; ordinate: interferon titre per ml after 24 hours

– – – – – Interferon titres in cells kept in contact with endotoxin for 24 hours (24 hr – control)

cultures (after 24 hours) were as high as in control tubes permanently containing endotoxin without change of the medium. These controls showed that the procedure of the medium exchange itself did not cause a loss of cells which could be responsible for the interferon losses. However, to be sure that all endotoxin capable of interferon stimulation was removed by medium exchange, a limit dilution of endotoxin was used in the experiments. This was found by constructing a dose-response curve as follows:

Peritoneal cells were treated with different dilutions of the endotoxin preparation at 26° C. After 24 hours, the resulting interferon titres were determined in L cells (Fig. 1). The endotoxin preparation used proved to be effective up to a dilution of $10^{-4.5}$. The interferon-stimulating capacity decreased by further dilution, and at $10^{-6.5}$ an interferon-stimulating effect could no more be demonstrated. In further experiments an endotoxin dilution of $10^{-4.5}$ was used, since after removal of medium containing this amount of endotoxin and addition of fresh medium to cells a dilution effect of at least 1 : 100 was achieved, which proved to be sufficient for elimination of the remaining interferon-stimulating capacity. Fig. 2 demonstrates the effect of removing the endotoxin from the medium on the release of interferon by peritoneal cells. It is evident that the contact of cells with endotoxin for 20 minutes elicited a maximal interferon release which could not be enhanced by further incubation of cells in the presence of endotoxin.

Temporal relationships between interferon release and incubation of cells at 26° C

Mouse peritoneal cells freshly derived from the peritoneal cavity were treated with endotoxin diluted to 10^{-2} immediately after transfer to tubes and incubated at 26° C. At different time intervals the tubes were transferred to 36° C and further incubated at this temperature. The interferon titres were determined after a total incubation time of 24 hours after addition of endotoxin to the cells. The titres were compared with those in control samples permanently incubated for 24 hours either at 26° C or at 36° C.

Table 1. The influence of preincubation of endotoxin-treated mouse peritoneal cells at 26° C in vitro on resulting interferon titres after transfer to 36° C

Time of preincubation at 26° C after addition of *E. coli* 0111 : B4 endotoxin (hours)	24 hours' yield of interferon after transfer of cells to 36° C (titre per ml)			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0	16	4	4	4—8
0.5		8	8	
1		16	16	8
2	64		32	8—16
3				32
4	64		64	64—128
8	64			
24	64—128	128	128	128

The results of four different experiments are summarized in Table 1. The transfer of cells to the supraoptimal temperature of 36° C did not influence the 24-hour interferon yield when the freshly derived peritoneal cells were incubated at 26° C in the presence of endotoxin for at least 4 hours after addition of endotoxin. The interferon titres in this case were comparable to those found after permanent incubation of the cell culture at 26° C,

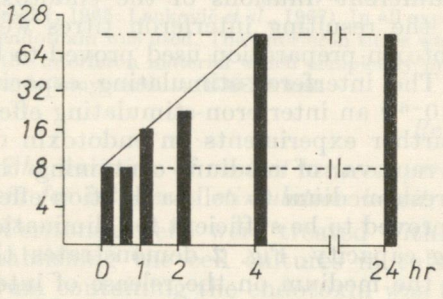

Fig. 3.

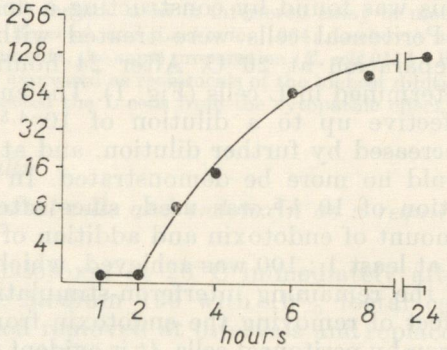

Fig. 4.

Fig. 3.

The influence of preincubation of endotoxin-treated cells at 26° C on the resulting interferon titres at 36°C

Abscissa: time of incubation of endotoxin treated cells at 26° C; ordinate: interferon titre per ml after 24 hours

Black columns: Cells preincubated at 26° C and then transferred to 36° C

-.-.-.- Cells incubated permanently at 26° C (26° C-control)

– – – – – Cells incubated permanently at 36° C (36° C-control)

Fig. 4.

Interferon production in mouse peritoneal cells at 26° C in vitro after stimulation with endotoxin
Abscissa: time of incubation; ordinate: interferon titre per ml

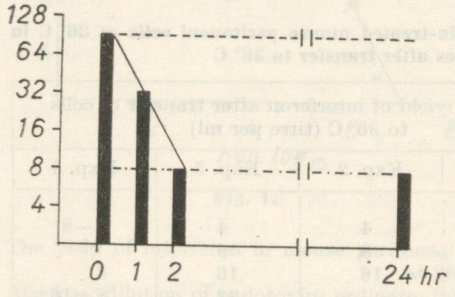

Fig. 5.

The influence of preincubation of endotoxin-treated mouse peritoneal cells at 36° C on the resulting interferon titres at 26° C

Abscissa: time of preincubation at 36° C; ordinate: interferon titre per ml after 24 hours

Black columns: Cells preincubated at 36° C and then transferred to 26° C

– – – – – Cells incubated permanently at 26° C (26° C-control)

-.-.-.- Cells incubated permanently at 36° C (36° C-control)

whereas a permanent incubation at 36° C yielded 3.5 $\log_2$ lower interferon titres. An incubation time of at least 1 hour at 26° C was necessary to obtain a measurable increase of the interferon yield after raising the temperature up to 36° C.

These relations are illustrated in Fig. 3. For comparison, Fig. 4 shows the course of interferon release from peritoneal cells after stimulation with endotoxin (dilluted 10^{-2}) at 26° C. As shown, the course of interferon release was comparable with the results already published by Lackovič *et al.* (1967) and Borecký and Lackovič (1968). The bulk quantity of interferon was released between the 4th and 8th hour after addition of endotoxin. However, in this period the incubation temperature of 26° C was no longer necessary for release, as can be seen in Fig. 3.

Fig. 5 summarizes the results of experiments which show the influence on the resulting interferon release of incubation of endotoxin-treated cells at 36° C before the transfer of tubes to 26° C. These experiments were carried out in a similar way as those presented in Fig. 3. Evidently, an incubation time of only 1 hour at 36° C, followed by a shift to the optimal temperature of 26° C up to 24 hours was sufficient to depress the interferon yield to the levels obtained in cells permanently kept at 36° C. Thus the necessity of an optimal temperature during a period when a significant interferon release cannot be demonstrated was confirmed. This time interval may be called "temperature-sensitive stimulatory phase".

Discussion

The interferon release by mouse peritoneal cells in vitro becomes irreversible after a short contact time of endotoxin with the cells (Fig. 2). The reason for this is not understood. Endotoxin may be bound to the cell surface via "receptors" (Springer *et al.*, 1966) and may penetrate into leukocytes (Mesrobeanu *et al.*, 1967). However, the binding capacity of the cell for endotoxin is probably limited (Fig. 1). The addition of specific antibodies with high avidity to endotoxin enhanced the interferon release by peritoneal cells in vitro (Borecký *et al.*, 1968). This effect could be due to an overcoming of the saturation limit by a better binding of the endotoxin-antibody complex by the cells, or by mitigating the toxicity of the preparation.

The temperature optimum of 26° C found for the interferon release by peritoneal cells in vitro after stimulation with endotoxin proved to be necessary only at the beginning of this event ("temperature-sensitive stimulatory phase"). A correlation seems to exist between the optimal interferon release temperature for mouse peritoneal cells and the loss of this ability in the course of cultivation in vitro. As demonstrated previously (Lackovič *et al.*, 1967), the loss of interferon releasing ability was temperature-dependent, too. Low temperatures like 4° C or 26° C delay it, while at 36° C the process progressed more quickly. A temperature shift to 36° C did not reduce the resulting interferon yield (Fig. 3), when a 4 hours' incubation at 26° C after addition of endotoxin preceded the temperature change. On the other hand, the bulk of interferon release seemed to occur after the 4 hours of incubation (Fig. 4). Therefore, one could assume that the cells may have lost their capability of being stimulated by endotoxin within these 4 hours. Consequently, at 36° C the loss would occur within 1 hour (Fig. 5), which means four times as quickly as at 26° C.

To test this assumption we determined once more the interferon yield in vitro in relation to preceding cultivation of peritoneal cells. The assay was carried out as previously described (Borecký and Lackovič, 1968). The data obtained are summarized in Table 2. It is evident that cells cultivated for 1.5 hours at 26° C, before treatment with endotoxin, produced 4.5 $\log_2$

Table 2. The influence of preincubation of mouse peritoneal cells at 26° C on their capacity to release interferon after subsequent endotoxin stimulation in vitro

Time of incubation at 26° C before addition of *E. coli* 0111 : B4 endotoxin to the cells (minutes)	Yield of interferon 24 hours after addition of *E. coli* 0111 : B4 endotoxin to the cells*
0	8.0
30	6.0
60	5.0
90	3.5
120	2.5

* Interferon titre given in $\log_2$ values per ml.

steps less interferon than those freshly derived and stimulated immediately after establishing the culture. Since peritoneal cells could not be stimulated by endotoxin to produce more interferon after 4 hours at 26° C, it could be expected that cells "aged" 1.5 hours at 26° C should be sensitive for only 2.5 additional hours. This conclusion should be applicable also for "fresh" cells, provided that the cells treated immediately after explantation with endotoxin were first incubated at 26° C for 2 hours and then transferred to 36° C for further incubation. Since at the latter temperature the sensitivity for endotoxin decreased four times more quickly, the temperature shift would cause a shortening of the "temperature-sensitive stimulatory phase" to 30 minutes. If true, the cells at 37° C could be stimulated by endotoxin for only 2.5 hours althogether.

This assumption was not confirmed, however. Fig. 3 shows that in this case the interferon yield was only 2.0 $\log_2$ steps lower than in cells permanently incubated at 26° C. This indicates necessity of further experiments.

An alternative explanation could be attempted by the assumption that peritoneal cells in vitro permanently loose by degradation a "preformed" interferon (or an interferon precursor) like that postulated by Ho and Breinig (1965) and Youngner *et al.* (1965). Supposedly, the loss would proceed more slowly at 26° C than at 36° C. Since interferon release was not detected during the first 2 hours after endotoxin addition to the cells (Fig. 4), this interval seems to be necessary for the release of preformed and stored interferon from the stimulated cell (wall?) components, or for endotoxin-induced transformation of the precursor into mature interferon. The prompter the degradation would go on up to this time and thereafter, the less resulting interferon would be released and vice versa. Therefore the preformed interferon (or the hypothetical "precursors") must be present

in the cells as long as they are able to release interferon on endotoxin stimulation. At 26° C this would be nearly 8 to 9 hours as can be seen in Fig. 4. Since after a 4 hours' incubation at 26° C the temperature can be shifted up to 36° C without impairing the resulting interferon yield (Fig. 3), at least 2 to 3 hours should pass away after the temperature change up to a complete degradation. There is only a slight interferon release detectable between the 6th and 8th hour (Fig. 5).

From this it could be deduced that the postulated degradation of preformed interferon would proceed at 36° C nearly two times as quickly as at 26° C. If the stimulation of interferon release at 26° C would be effective only during 2 hours after addition of the bacterial stimulator, then the extent of the postulated degradation up to this time point might be of decisive importance for the resulting interferon yields. Peritoneal cells incubated after addition of endotoxin for the first hour at 36° C and then shifted to 26° C (Fig. 5) are expected to retain a reduced quantity of "preformed" interferon. If the rate of degradation at 36° C within the first hour would be twice as quick as at 26° C, the resulting interferon yield should have been reduced to an extent comparable to that obtained by fresh peritoneal cells preincubated at 26° C for 1 hour before addition of endotoxin. As evident from the corresponding data in Fig. 5 and Table 2, the interferon yields were in both cases 3.5 and 3.0 $\log_2$ units lower than when fresh cells were immediately treated with endotoxin and kept at 26° C permanently (24 hours). Moreover, the interferon titres determined after a permanent incubation at 36° C were as high as those obtained after an incubation for 1 hour at this temperature followed by a shift to 26° C (Fig. 5). Following the hitherto made assumptions, the interferon titres determined in the first case should be lower than in the second one. Since this was not the case, it is suggested that endotoxin acts as a stimulator somewhat earlier at 36° C than at 26° C. The observed delay of the effective endotoxin stimulation of cells could be caused by the time required for establishing an effective cell contact (phagocytosis?). This event seems to be temperature-dependent also and would take place more quickly at 36° C than at 26° C. In accordance with the above-made assumptions, peritoneal cells treated with endotoxin at 26° C for 2 hours and then transferred to 36° C should have lowered final (24 hours) titres indicating a decay of postulated preformed interferon to an extent of 3 hours at 36° C (compared to about 6 hours at 26° C after the first 2 hours' period). The resulting interferon titres were only as high as the titres obtained from cells incubated at 26° C for 5 hours. The corresponding data from Figs 3 and 4 are comparable also from this point ofview.

Consequently, the temperature optimum could be just the point at which the process of phagocytosis of endotoxin (or establishment of other effective contact with the cell) and the process of the postulated degradation of "preformed" interferon (or its precursor) correlate in optimal way for interferon release. Lower temperatures would prolong the first event strikingly, whereas higher ones would enhance the degradation of precursor too much.

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