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TRANSMISSION OF DENGUE VIRUS-INDUCED SUPPRESSOR SIGNAL FROM MACROPHAGE TO LYMPHOCYTE OCCURS BY CELL CONTACT

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Summary.—We have observed that live macrophages play an obligatory role in transmission of dengue type 2 virus (DV)-induced suppressor signal to a subpopulation of T lymphocytes. The present study was undertaken to resolve whether transmission of the suppressor signal is mediated by a soluble factor or by direct cell-to-cell contact. It was observed that the suppressor factor (SF) remains adsorbed on the surface of macrophages and can be retrieved from them completely by DV-stimulated spleen cells kept in contact with them. Suppression of DV-specific IgM plaque-forming cells (PFC) in spleen-cell cultures does not occur when SF-adsorbed macrophages are separated from them by cell-impermeable membranes. Culture fluid of SF-adsorbed macrophages have no suppressor activity. The suppression of PFC occurs only when SF-adsorbed macrophages remain in contact with DV-stimulated spleen-cell cultures. Thus transmission of suppressor signal from SF-adsorbed macrophages to lymphocytes occurs only by physical contact of the plasma membranes of the interacting cells.

ANTIGEN-SPECIFIC IMMUNOSUPPRESSION during dengue virus (DV) infection is mediated by a 2-step mechanism. In the first step DV stimulates a subpopulation of suppressor T lymphocytes (TS_1) to produce a suppressor factor (SF) (Chaturvedi *et al.*, 1978; Tandon, Chaturvedi and Mathur, 1979). The SF is produced in the spleens of DV-infected sick mice and is a very-low-mol.-wt, heat-labile, trypsin-resistant substance which is unstable at acidic and alkaline pH (Chaturvedi and Shukla, 1981). In the 2nd step SF recruits a subpopulation of T lymphocytes (TS_2) which produces prostaglandin, which finally suppresses DV-specific IgM, plaque-forming cells (PFC) (Chaturvedi, Shukla and Mathur, 1981; Shukla and Chaturvedi, 1981). The Ly phenotype of TS_1 and TS_2 is Ly 23^+ (Shukla, Dalakoti and Chaturvedi, 1982). We have further observed that the presence of live macrophages is obligatory for the transmission of suppressor signal by SF to TS_2. SF is adsorbed on to the macrophages or macrophage-like cells and these cells pass the signal to TS_2 *in vitro* as well as *in vivo* (Chaturvedi, Shukla and Mathur, 1982; Shukla and Chaturvedi, 1982). The transmission of suppressor signal to TS_2 cells can be mediated either by cell-to-cell contact or through secretion of a soluble intermediate factor. The present study was undertaken to resolve these questions. The findings presented in this paper indicate that SF-adsorbed macrophages and TS_2 cells require close physical contact for transmission of suppressor signal.

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MATERIALS AND METHODS

Animals.—Inbred adult Swiss albino mice obtained from the colony maintained in this department were used.

Virus.—Dengue type 2 virus (DV) strain P23085 was used in a dose of 1000 LD_{50}. Details of the virus has been described elsewhere (Chaturvedi, Tandon and Mathur, 1977; Chaturvedi *et al.*, 1978; Chaturvedi, Bhargava and Mathur, 1980*a*; Chaturvedi, Dalakoti and Mathur, 1980*b*).

Suppressor factor (SF).—Details of the preparation of SF has been described elsewhere (Chaturvedi and Shukla, 1981). In brief, a 10% w/v homogenate of DV-infected sick-mouse spleens was prepared in phosphate-buffered saline (PBS), pH 7·0 in a MSE tissue homogenizer. It was centrifuged at 2000 *g* for 10 min and the clear supernate was again centrifuged, at 103,500 *g*, for 3 h. The supernatant obtained was stored in small aliquots at −20° and used as SF. Similarly prepared homogenate of normal mouse spleen (NH) was used in controls in every experiment and was found to have no suppressor activity.

Preparation of SF-adsorbed macrophages.—Mouse peritoneal macrophages collected in heparinized MEM (Earles) containing 10% foetal calf serum (FCS) were layered in glass Petri dishes and incubated at 37° for 2 h in the presence of CO_2. Non-adherent cells were washed off. On the macrophages cell sheet thus obtained 0·5 ml SF (10^{-2}) was layered and allowed to adsorb for 1 h at 37°. Unadsorbed SF was removed by gently washing the cell sheet 3× with medium containing 5% FCS. The cells were scraped off if required or the SF-adsorbed cell sheet was used as such. For controls normal mice spleen homogenate (NH) was adsorbed on to macrophage cell sheets (Chaturvedi *et al.*, 1982; Shukla and Chaturvedi, 1982).

Preparation of spleen cell cultures.—Normal mouse spleen cells were cultured in MEM containing HEPES buffer, 10% FCS and 5×10^{-5}M 2-mercaptoethanol as described elsewhere (Shukla and Chaturvedi, 1981). The cells were cultured in 5 cm glass Petri dishes, each containing 4 ml cell suspension (5×10^6 cells/ml). Cultures were incubated at 37° in an atmosphere of 5% CO_2.

Experimental protocol.—The experimental protocol was the same as that described by Shukla and Chaturvedi (1981). In brief, normal mouse spleen-cell cultures were inoculated with DV at 0 h in a dose of 10^3 LD_{50} followed 24 h later by inoculation of SF, or exposure to SF-adsorbed macrophages. On the 3rd and 4th day of culture DV-specific IgM PFC were counted by the localized-haemolysis-in-gel technique of Jerne and Nordin (1963) as described elsewhere (Chaturvedi *et al.*, 1977; Tandon and Chaturvedi, 1977). The number of PFC per 2×10^6 nucleated spleen cells are presented. Cultures were set in triplicate and each experiment was repeated twice or 3 times. For controls NH or DV only was inoculated in cultures. Mean values with standard deviations are presented after deducting background PFC. The data have been analysed using Student's *t* test for *P* values. A *P* value of less than 0·05 was considered significant.

RESULTS

Absence of suppressor activity in culture fluid of SF-adsorbed macrophages

This experiment was done to find out if SF induces macrophages to produce an intermediary product for transmission of suppressor signal to T lymphocytes. SF-adsorbed macrophages were cultured for 24 h and the culture fluid was then decanted, leaving behind the cell sheet. Suppressor activity of the cell-free culture fluid (TCF) was screened by inoculation in DV-stimulated spleen-cell cultures. After removal of TCF the macrophage cell sheet was washed 3 times. To assay its suppressor activity, it was covered with DV-stimulated spleen-cell cultures for 1 h at

TABLE I.—*Absence of suppressor activity in the culture fluid of SF-adsorbed macrophages*

Spleen-cell cultures* exposed to	PFC/2×10^6 spleen cells: Number ± s.d.	Suppression (%)
TCF of SF-adsorbed MØ	716 ± 37	8
TCF of NH-adsorbed MØ	715 ± 29	8
SF-adsorbed MØ	419 ± 27	46
NH-adsorbed MØ	697 ± 23	10
Nil	776 ± 30	0

* Mouse spleen-cell cultures were inoculated with DV 1000 LD_{50} at 0 h followed 24 h later by exposure to SF-adsorbed macrophage cell sheets or inoculation of culture fluid (TCF) of SF-adsorbed macrophages. DV-specific IgM PFC were counted on Days 3 and 4. (Means ± s.d.).

37°; the spleen cells were then decanted and cultured further for PFC counting (Chaturvedi *et al.*, 1982). The data presented in Table I show that the suppression of PFC by TCF was 8%, while that by SF-adsorbed macrophage cell sheet was 46%. Neither the TCF from NH-adsorbed macrophages nor the NH-adsorbed macrophage cell sheet had any suppressor activity (Table I). This showed that neither SF nor any other product is released from SF-adsorbed macrophages. But this did not rule out the possibility of the production of a highly labile, short-lived intermediary product. The following experiments were therefore carried out.

Suppressor activity of SF-adsorbed macrophages separated from DV-stimulated cultures by cell-impermeable membranes

SF-adsorbed macrophages and DV-stimulated spleen-cell cultures were kept separate by a dialysing membrane. On Days 3 and 4 PFC were counted and the findings are summarized in Table II. It was observed that SF-adsorbed macrophages did not mediate suppression through the dialysing membrane, as the suppression of PFC was 11%. On the other hand SF alone mediated maximum suppression of PFC through the dialysing membrane. Macrophages alone or those adsorbed with NH had no effect on the PFC count when separated by a dialysing membrane (Table II).

Since substances of very low mol. wt can only pass through a dialysing membrane, the above experiment was repeated keeping DV-stimulated spleen-cell cultures separated from the SF-adsorbed macrophages by a Millipore membrane (pore size 0·45 μm, Millipore Filter Corporation, Boston, Mass.). The findings of these experiments are summarized in Table II. It was noted that SF mediated 54% suppression of PFC through the Millipore membrane, while that mediated by SF-adsorbed macrophages was 15%. Macrophages alone or those adsorbed with NH had no effect on PFC count when separated by a Millipore membrane (Table II).

Retrieval of suppressor signal from SF-adsorbed macrophages

We have observed that PFC are suppressed when DV-stimulated spleen-cell cultures are co-cultivated or incubated for 1 h at 37° with SF-adsorbed macrophages (Chaturvedi *et al.*, 1982). In the present series of experiments an effort was made to find if the suppressor signal is depleted in SF-adsorbed macrophages by repeated exposure to DV-stimulated spleen-cell cultures. For this the SF-adsorbed macrophage cell sheet was layered with spleen-cell cultures stimulated with DV 24 h earlier, and incubated at 37° for 1 h; the spleen cells were then decanted and cultured for PFC count. The macrophage sheet was washed 3 times and layered over

TABLE II.—*SF-adsorbed macrophages fail to transmit suppression across cell-impermeable membranes*

	Cell-impermeable membrane			
	Dialysing membrane		Millipore membrane	
Spleen-cell cultures* separated from	PFC count	Suppression (%)	PFC count	Suppression (%)
SF	438 ± 62	44	360 ± 28	54
SF-adsorbed MØ	693 ± 30	11	660 ± 30	15
NH-adsorbed MØ	682 ± 39	12	680 ± 49	12
Untreated MØ	709 ± 37	9	744 ± 29	4
Nil	776 ± 30	0	776 ± 30	0

* Mouse spleen-cell cultures were inoculated with DV 1000 LD_{50} at 0 h. At 24 h to these cultures were added chambers of cell-impermeable membranes containing 5×10^6 macrophages coated with SF or NH. On Days 3 and 4 DV-specific IgM PFC were counted in spleen-cell cultures. Mean values ± s.d. per 2×10^6 cells are presented.

TABLE III.—*SF remains on cell surface and can be completely retrieved*

Spleen-cell cultures* exposed to	PFC/2 × 10^6 spleen cells	
	Number	Suppression (%)
Untreated MØ	744 ± 35	4
SF-adsorbed MØ—1st retrieval	419 ± 27	46
SF-adsorbed MØ—2nd retrieval	638 ± 19	18
SF-adsorbed MØ—3rd retrieval	725 ± 27	7
Homogenate of MØ after 3rd retrieval	784 ± 137	− 1
NH-adsorbed MØ	697 ± 23	10
Homogenate of NH-adsorbed MØ after 3rd retrieval	711 ± 46	8
Nil	776 ± 30	0

* Mouse spleen-cell cultures were inoculated with DV 1000 LD_{50} at 0 h. After 24 h these cultures were layered over SF-adsorbed macrophage sheets for 1 h at 37°. The cultures were then decanted and cultured for PFC counts on Days 3 and 4. The SF-adsorbed macrophage cell sheet was washed and re-exposed to spleen-cell cultures twice more. Mean values of PFC ± s.d. are presented.

with a second set of DV-stimulated spleen-cell cultures. After exposure for 1 h at 37° the cells were decanted and cultured. The macrophage cell sheet was layered over with the 3rd set of DV-stimulated spleen-cell cultures after washing 3 times. The retrieval of suppressor signal from the macrophages by spleen-cell cultures was assayed by counting PFC in spleen-cell cultures on Days 3 and 4 after DV stimulation. To screen for the interiorized suppressor signal the macrophage cell sheet remaining after the 3rd retrieval was washed 3 times and then harvested with the help of a rubber-tipped glass policeman. The cells were homogenized in PBS and the suppressor activity of the homogenate was assayed by inoculating it in DV-stimulated spleen-cell cultures.

The data summarized in Table III show that in the first set of cultures exposed to SF-adsorbed macrophages the suppression of PFC was 46%. In the 2nd set of cultures the suppression of PFC was reduced to 18%, while with the 3rd set of cultures the suppressor signal could not be retrieved as the suppression was negligible in this set, being 7%. The suppressor activity was absent in the homogenate of macrophages prepared after 3 exposures to DV-stimulated spleen-cell cultures. Exposure of DV-stimulated spleen-cell cultures to untreated macrophages or NH-adsorbed macrophages of homogenate of the latter had no effect on the PFC count (Table III).

DISCUSSION

Macrophages play an important role in a variety of immunological phenomena but the precise mechanism of their action is poorly understood. One such mechanism is the transmission of antigen-specific signals to T lymphocytes. Transmission of signals from one cell to another can be mediated either through secretion of a soluble mediator or by close contact of the plasma membranes of the interacting cells. Erb and Feldmann (1975) have presented evidence for the generation of specific secretory helper-cell activity in mice. However, the findings of the present study demonstrate that SF-adsorbed macrophages neither release SF nor any other soluble product which may mediate suppression of DV-specific IgM PFC. This conclusion has been drawn from 2 experiments, *viz.*: SF-adsorbed macrophages fail to transmit suppressor signal when separated from spleen-cell cultures through cell-impermeable membranes; and absence of suppressor activity in the culture fluid of SF-adsorbed macrophages.

We have observed that uptake of SF is similar in both live and killed macrophages, but it can be presented to target T lymphocytes only by live macrophages. Thus SF uptake is a passive phenomenon but its presentation requires active cell metabolism (Chaturvedi *et al.*, 1982). The findings of the present study show that the adsorbed SF remains only at the surface of the macrophages and can be retrieved

from them completely. It is not known how SF remains tightly bound to the surface of macrophages but we do know that the SF is adsorbed only on syngeneic macrophages and suppressed DV-specific PFC in syngeneic spleen cells (Chaturvedi *et al.*, 1982). Thus binding of SF through major histocompatibility complex (MHC) products can be a strong possibility and remains to be further investigated.

The second mechanism through which the signal can be transmitted is by close contact of plasma membranes of the macrophages and T lymphocytes. Findings of the present study show that this can be the mechanism of the transmission of the suppressor signal as it is possible only when the cells are in close physical contact, and when the cells are separated by a cell-impermeable membrane the suppressor signal is not transmitted. Rosenthal, Barcinski and Rosenwasser (1978) have described 3 types of physical interactions between macrophages and lymphocytes for transmission of antigen-specific signal. Active metabolism, divalent cations and a trypsin-sensitive sites are required on macrophages in the first type. The binding in this reaction can be with T or B cells and is reversible (Lipsky and Rosenthal, 1973).The second type of binding is antigen-specific, not easily reversible, and is dependent upon the presence of antigen and a sharing of MHC-linked gene products on macrophages and lymphocytes (Lipsky and Rosenthal, 1975). The third type of binding is non-antigen-specific, easily reversible and occurs when lymphocytes cluster about the antigen-specific central lymphocytes (Werdelin, Braendstrup and Pedersen, 1974). We have observed that SF remains firmly bound to macrophages and mediates antigen-specific signal in syngeneic cells and not in allogeneic cells (Chaturvedi *et al.*, 1982); therefore it is likely that the binding of macrophage to lymphocyte in our model is of the second type of Rosenthal *et al.* (1978).

The findings of the present study thus show that transmission of suppressor signal from macrophage to lymphocyte is a surface phenomenon and occurs only by physical contact of the plasma membranes of the interacting cells. With the available data it is not possible to say if SF undergoes any change in the macrophage or is presented in its original form in optimum amount to the T lymphocytes.

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