

## Effect of Low Molecular Factors Released During Allogeneic Interactions of Leucocytes or Brain Cells on PHA- and LPS-induced DNA Synthesis in Lymphocytes

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### Summary

Male (A/Ph and C3H/Cbi/BOM) mice were used in our experiments. The cell-free supernatants of spleen mixed leucocyte cultures and allogeneic brain cortex cells were collected upon short-term incubation and tested in a comparative study. Spleen and brain low molecular factors were fractionated (ultrafiltration and gel column chromatography) and assayed for their activity in mitogen-induced  $^3\text{H}$ -thymidine incorporation into spleen lymphocytes.

The specific release of the allogeneic spleen fraction (ASF-2) has been observed. A non-specific stimulating effect of ASF-2 on PHA- and LPS-activated spleen lymphocytes was shown. In contrast, a non-specific suppressive effect of brain fraction on PHA- and LPS-stimulated lymphocytes was demonstrated. The opposite effects of spleen and brain factors can be connected with their different composition.

The lymphocyte cultures were not affected by spleen or brain factor, when cultured without the addition of mitogen.

Positive and negative regulation or modulation of triggering events in lymphocyte activation by these factors can be suggested.

Numerous biologically active substances (mainly of high molecular weight) have been reported in supernatants (SUPs) of allogeneically stimulated leucocytes (MLC). Since these substances – lymphokines – are stimulatory and others are inhibitory for lymphocytes, it is generally accepted that they are important regulators of model immune reactions *in vitro* (1).

The aim of this report was to estimate the possible regulatory or modulatory activity of low molecular fractions of SUPs upon short-term spleen MLC and to compare these fractions with SUP fractions of short-term allogeneic interaction of brain cortex cells *in vitro*.

In previous studies the marked metabolic changes have been found in the mixed brain reaction (MBR) and the role of the released low molecular factor in these changes has been suggested (2, 3, 4, 5). It was of considerable interest to examine adequate SUP fraction of MLC, isolated by the same procedure as MBR-factor, in comparative experiments.

### Material and Methods

Male A/Ph and C3H/Cbi/BOM mice (8 to 12 week-old) bred under specific pathogen-free conditions were obtained from Velaz breeding facilities (Šumice, Czechoslovakia).

MLC SUPs. Spleens were teased and single-cell suspensions were prepared as previously described (6). The unseparated spleen cells were then resuspended to appropriate cell concentration in tissue culture medium (MEM, Gibco) and incubated as indicated below.

Two-way MLC consisting of equal parts of allogeneic spleen cells was the SUP source. Cells ( $1.10^7/\text{ml}$ ) in MEM medium without serum were incubated in closed Erlenmeyer flasks in plastic bags for 4 hr at  $37^\circ\text{C}$  in humidified atmosphere of 5%  $\text{CO}_2$  in air.

MBR SUPs. Cell suspensions were prepared by teasing of brain cortex fragments through different nylon sieves in isotonic medium with purified polyvinylpyrrolidone and then washed three times (7). Allogeneic cell suspensions were composed of equal parts of cells (8 mg cell protein/ml) in isotonic saline medium with 10 mM glucose. Cells were incubated in closed Erlenmeyer flasks in ice-cold bath for 30 minutes followed by incubation in shaking water-bath at  $37^\circ\text{C}$  for 60 minutes. The allogeneic

cell suspensions were centrifuged at 12,000xG for 15 min, and SUPs were immediately frozen at  $-25^{\circ}\text{C}$  and kept overnight. The low molecular factor was released, when allogeneic, but not when syngeneic brain cortex cells were mixed and incubated.

Fractionation of SUPs. Thawed SUP was pressure-dialyzed in Amicon ultrafiltration cell with UM-10 Diflo membrane (Amicon Corp.) at  $+3^{\circ}\text{C}$ . Low molecular part of SUP ( $< 10,000\text{ D}$ ) was lyophilized. Powder, dissolved in eluant, was applied on column of Biogel P-2, particle diameter 37–75  $\mu\text{m}$  (Bio. Rad Labs) with 1M  $\text{CH}_3\text{COOH}$  as eluant. The absorbance (A) of each fractions was measured at different wave lengths: at 280 nm (maximal aromatic amino acid absorption), at 250 nm (high nucleotide absorption) and at 310 nm (presence of metal-bound ?). The fractions were lyophilized and stored until used at  $+4^{\circ}\text{C}$ .

Mitogens. Phytohemagglutinin M (PHA, Difco), final dilution 1:100 per well and lipopolysaccharide (LPS) isolated from LT2 Salmonella typhimurium (provided by Dr. J. Hofman from our institute) in amount of 20  $\mu\text{g}$ /well were used.

Assay of mitogenic activity. Spleen cells ( $1.2 \times 10^6/0.25\text{ ml}$ ) of C3H/Cbi/BOM mice were cultured in microplates (M29 ART, Cooke) with or without mitogen. Cultures were pulsed with  $^3\text{H}$ -thymidine (1  $\mu\text{Ci}$ /well; 19.3 Ci/mmol) for the final 8 to 12 hr of the 3 day incubation. Cells were harvested on a multiple semiautomated sample harvester (Prague). The radioactivity incorporated in trichloroacetic acid-insoluble material (counts per minute) was measured in a liquid scintillation counter (Isocap 300, Nuclear Chicago). For further details see (6). Each value represents the arithmetical mean of six measurements  $\pm$  s.e.m.

Values of incorporated radioactivity into lymphocyte cultures: control values (without mitogen) 1,010 to 1,410 c.p.m./culture, with mitogen (PHA or LPS) 8,960 to 11,394 c.p.m./culture.

## Results and Discussion

Elution profiles of low molecular parts of MLC-SUPs and MBR-SUPs were obtained after gel filtration on column of Biogel P-2 (exclusion limit 2,600 D) (Fig. 1A and 2A). The spleen and brain fractions, eluted in elution volume of 80–100 ml were assayed for their effect on mitogenic activity of spleen cells. Two spleen fractions ASF-1 and ASF-2 (allogeneic spleen fraction) were found to be in the determined region. The elution volume of ASF-1 was rather similar to that of control SSF (syngeneic spleen fraction) (Fig. 1C), even if the effects of these fractions on mitogen-stimulated lymphocytes were not identical (Fig. 1B and 1D). In ASF-1 specifically and non-specifically released material could be included. On the other hand, ASF-2 may be an alloantigen specific released fraction with enhancing effect on PHA- and LPS-stimulated lymphocytes.

The suppressive effect of ABF-1 and ABF-2 (allogeneic brain fraction) on mitogen-activated lymphocytes has been found (Fig. 2B). These findings may indicate a similar blocking effect of the brain fractions on the metabolism of activated cells.

Elution profiles of fractions were measured at different wave lengths. From the different absorbance peaks (at 250, 280 and 310 nm) of spleen and brain factors, the presence of different substances in isolated fractions may be suggested. These data can be related to the opposite effect of fractions on mitogen-activated lymphocytes.

Although each of these fractions added to lymphocyte cultures without mitogen was ineffective (Table 1). These data also serve as a control of the possible cytotoxicity of fractions.

Numerous factors have been reported in supernatants of allogeneically activated lymphocytes, these included migration inhibition factor, blastogenic factor, allogeneic (effect) factor, colony-stimulating factor, macrophage activation factor, potentiating factor, inhibitory factor, lymphotoxin, leukotactic factor and auto-stimulating factor as reviewed by Roehm (8), thymocyte stimulating factor (9) and helper

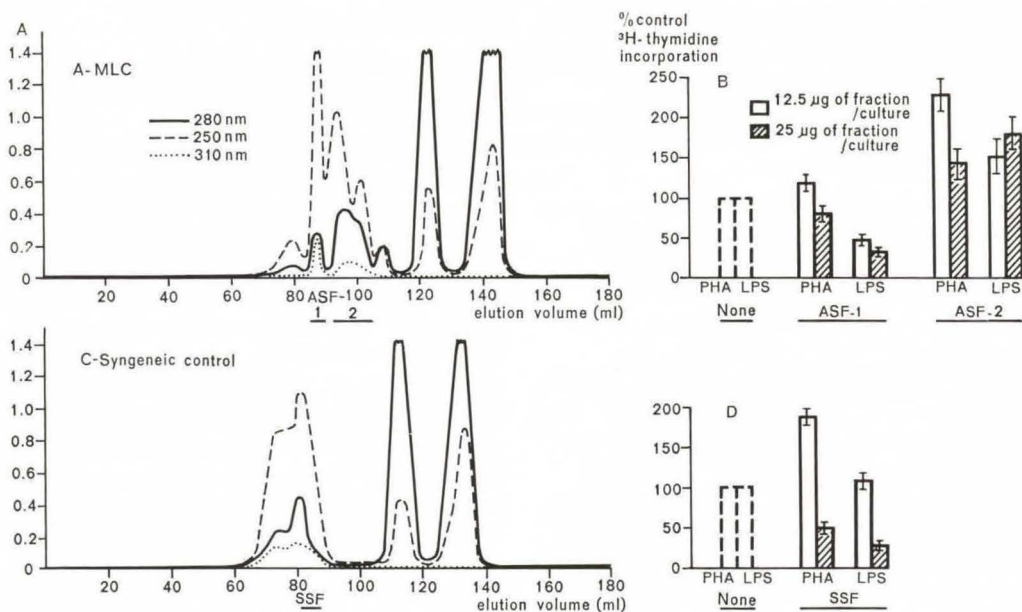


Fig. 1 Fractionation of low molecular part of spleen supernatants by gel filtration on Biogel P-2 and effect of fractions on  $^3\text{H}$ -thymidine uptake by mitogen-activated lymphocytes.

Gel filtration — MLC supernatant (1A), syngeneic lymphocyte culture supernatant (1C). Effect of fractions on isotope incorporation (1B, 1D). PHA- or LPS-stimulated incorporation without fraction was taken as 100% (control incorporation). The changes of incorporation above or under this level were due to the action of different fraction concentrations. Each result represents arithmetical mean of six measurements  $\pm$  s.e.m.

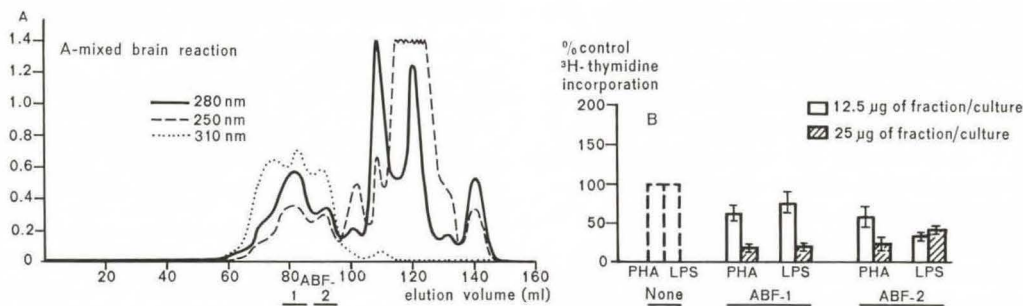


Fig. 2 Biogel P-2 gel filtration of low molecular part of supernatant of allogeneic brain cortex cell reaction and effect of fractions on mitogen-activated  $^3\text{H}$ -thymidine uptake by lymphocytes. Gel filtration (2A), effect of fractions on isotope incorporation (2B), other details — see Fig. 1

mediators (10). Each of these factors plays its biological role in many immunological phenomena. These factors are isolated from supernatants in later phase of allogeneic leucocyte interaction and they have a higher molecular weight. Recently, the factors (<10,000 D) were obtained from MLC supernatants

after 72 hour incubation, which stimulate leucine incorporation in tissue cultures and induce splenomegaly when injected in vivo (8).

We supposed, that rapid release of low molecular spleen MLC factor and its promoting effect on mitogen-activated spleen cells may be

Table 1 Effect on lymphocyte culture of low molecular factors released during allogeneic (ASF) or syngeneic (SSF) spleen cell interaction or allogeneic brain cortex (ABF) cell interaction

	$\mu\text{g}$ of factor	
	12.5	25
None	100.0	100.0
ASF-1	108.8 $\pm$ 5.0	109.9 $\pm$ 5.5
ASF-2	96.2 $\pm$ 7.9	98.2 $\pm$ 8.1
SSF	109.4 $\pm$ 3.4	87.7 $\pm$ 6.2
ABF-1	92.0 $\pm$ 8.9	102.2 $\pm$ 9.7
ABF-2	119.9 $\pm$ 7.4	115.6 $\pm$ 9.6

The results are expressed as % of control  $^3\text{H}$ -thymidine incorporation/well. Each result represents the mean of six measurements  $\pm$  s.e.m.

associated with a possible role of ASF-2 factor in triggering mechanisms of lymphocyte activation. The opposite effect of MBR fraction was observed, but a release of biologically active substances into supernatant during the early phase of allogeneic brain interaction is evident. These observations suggest the possibility that spleen (brain) factor might serve to modulate or regulate both B-cell and T-cell mediated immune responses.

Further analyses of rapidly released low molecular factors and their relationship to sequence of immune reactions is valuable (8, 11) and a correlation between analytically characterized factors and their effect on metabolic reactions of responder cells will be desired.

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