

## Взаимодействия между С-реактивным белком, сывороточным Р-компонентом амилоида и интерлейкином-8 и их влияние на функции нейтрофилов

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В первые часы воспалительной реакции возрастает синтез белков острой фазы воспаления - С-реактивного белка (СРБ) и сывороточного Р-компонента амилоида (РКА) - и одного из провоспалительных цитокинов - интерлейкина 8 (ИЛ-8). Нейтрофилы (Нф) первыми появляются в очаге воспаления и представляют один из наиболее ранних защитных барьеров организма. Важными показателями функциональной активности Нф являются их миграция и адгезия к эндотелиальным клеткам и белкам внеклеточного матрикса. ИЛ-8 влияет на трансэндотелиальную миграцию Нф, повышая плотность  $\beta 2$ -интегринов и их авидность к ICAM-1 и ICAM-2. Влияние белков острой фазы воспаления, в частности, СРБ и РКА, на экспрессию адгезионных рецепторов CD11b и CD18 и адгезию Нф к белкам внеклеточного матрикса не изучено; не охарактеризовано и их совместное действие с ИЛ-8. Ранее нами и другими авторами было показано, что СРБ может взаимодействовать с РКА и ИЛ-8, однако не было выяснено, как влияют эти взаимодействия на адгезию и миграцию Нф и на экспрессию адгезионных молекул на этих клетках.

В настоящей работе с помощью иммуноферментного метода было показано, что ИЛ-8 связывается с сорбированным на пластике СРБ. Методом ультрацентрифугирования не удалось показать взаимодействие этих белков в растворе. Таким образом,

условием связывания СРБ и ИЛ-8 являлась иммобилизация СРБ. ИЛ-8 связывался также с иммобилизованным РКА, но это взаимодействие носило более слабый характер.

Оказалось, что СРБ, РКА и ИЛ-8 являются хемоаттрактантами для Нф здоровых доноров, однако теряют хемоаттрактантные свойства при сочетанном действии. Экспрессию CD11b, CD18 и рецепторов фибронектина исследовали методом проточной цитофлуориметрии. Было установлено, что белки острой фазы воспаления изменяют экспрессию интегринов CD11b/CD18 и рецептора фибронектина ( $\alpha 5\beta 1$ -интегрина) на поверхности Нф. РКА обнаруживал свойства активатора адгезионных процессов, усиливая экспрессию CD11b/CD18 и рецепторов фибронектина. Комбинации белков, в состав которых входил РКА, также усиливали все процессы, способствующие адгезии. СРБ и ИЛ-8 понижали экспрессию фибронектинового рецептора. СРБ повышал экспрессию лишь одного из рецепторов (CD18), а в комбинации с ИЛ-8 и РКА частично нейтрализовал активность этих белков. При действии комбинаций РКА+СРБ или СРБ+ИЛ-8 наблюдался нейтрализующий эффект белков по отношению друг к другу.

Таким образом, было показано, что белки острой фазы воспаления СРБ и РКА влияют на адгезию и миграцию Нф, а в иммобилизованном состоянии могут связывать провоспалительный цитокин ИЛ-8. Это приводит к частичной нейтрализации биологической активности как пентраксинов, так и ИЛ-8. Следовательно, СРБ, РКА и ИЛ-8 могут выступать в качестве как про-, так и противовоспалительных факторов. Можно предполагать, что взаимодействие между белками острой фазы воспаления и ИЛ-8 регулирует процесс трансэндотелиальной миграции нейтрофилов в ткань и этапы развития воспалительной реакции.

**Ключевые слова:** пентраксины, С-реактивный белок, сывороточный Р-компонент амилоида, интерлейкин-8, фибронектин, стрептолизин О, интегрины, нейтрофилы человека, миграция, адгезия

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# Interactions of C-Reactive Protein and Serum Amyloid P Component with Interleukin-8 and Their Role in Regulation of Neutrophil Functions

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C-reactive protein (CRP) and serum amyloid P component (SAP) are acute phase proteins, whose concentrations increase within 24 h of inflammation along with concentration of IL-8. Polymorphonuclear neutrophil leukocytes (PMNs) form the earliest barrier protecting an injured organ during acute phase response. The aim of present work was to study interactions between CRP, SAP and IL-8, and to estimate the role of these interactions in regulation of neutrophil transendothelial migration. The results have shown that IL-8 binds to immobilized but not to free CRP. Binding of IL-8 to immobilized SAP was less strong. SAP like IL-8 increased CD11/CD18 integrin expression. IL-8 did not abolish the effect of SAP, and the mixture of IL-8 and SAP has stimulated CD11/CD18 expression. CRP upregulated CD18 but not CD11b expression. Under simultaneous action of CRP and IL-8, the stimulatory effect on CD11b and CD18 was abolished. The expression of fibronectin receptor was reduced by either IL-8 or CRP but increased by SAP. Effect of each protein was downregulated after following preincubations: CRP+SAP, CRP+IL-8 or SAP+IL-8. The mixtures of CRP with SAP, CRP with IL-8 or SAP with IL-8 showed no chemotactic activity, although each of the proteins was chemoattractive. Thus, acute phase proteins and IL-8 can act as anti-inflammatory factors upon binding each other. In summary, CRP and SAP influence PMN adhesion, migration and expression of CD11b/CD18 and fibronectin receptors, and can modulate the action of IL-8 on PMN attachment to endothelium and fibronectin, and on PMN traffic through the extracellular matrix during transendothelial migration.

## INTRODUCTION

The earliest physiological changes, revealing after an inflammatory stimulus are the expression of inflammatory cytokines and the increase of serum concentrations of acute phase reactants. These early molecules regulate further

development of inflammation. C-reactive protein (**CRP**) is a major acute phase protein in human, while its homologue, serum amyloid P component (**SAP**), shows less dramatic increase. It has been shown that SAP and CRP influence activity of the majority of cell types of immune system, including polymorphonuclear leukocytes (**PMN**). CRP and SAP stimulate PMN oxygen metabolism [1], induce degranulation of PMNs and release of proteases, upregulate adhesion and migration [2], and activate synthesis of certain cytokines [3]. PMN extravasation into inflamed or injured areas involves interaction

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with endothelial cells via surface adhesion molecules, and includes the following stages: (i) light attachment to vascular wall and rolling, (ii) tight attachment, and (iii) penetration through the vascular wall and moving in extracellular matrix toward the site of inflammation. Initial attachment of PMNs to endothelium and movement along its surface is mediated by L-selectin (CD62L). The CD11/CD18 integrin complex is largely responsible for tightening of adhesion and transendothelial emigration of neutrophils. PMN traffic through extracellular matrix is regulated by other cell surface integrins and chemotactic factors released by damaged tissue. One of the  $\beta$ 1-integrins involved is the classic receptor for fibronectin ( $\alpha$ 5 $\beta$ 1 integrin). It is constantly expressed on neutrophil surface, although can be modulated by inflammatory environment. Chemotactic attraction of PMNs to endothelium is provided by IL-8 synthesized by activated endothelial cells as well as by other molecules activating PMNs and facilitating their movement through extracellular matrix. IL-8 also acts to enhance the expression of  $\beta$ 2-integrins on PMNs and to increase their binding to endothelial ICAMs.

Despite the abundant data concerning the role of cytokines, integrins and selectins in transmigration of PMNs, there is a substantial gap in knowledge about the role of acute phase proteins in this process. Little or no data are available on individual or combined effects of CRP, SAP and IL-8 on PMN functions such as an expression of CD11/CD18, receptors for IL-8 and fibronectin (FN), an adhesion to extracellular matrix (particularly to fibronectin), and a migration rate. The aim of the present research was to study the effects of acute phase proteins (separately and in concert with IL-8) on these PMN activities. Additional motivation has come from our previous observations that binding of CRP to SAP inactivates both proteins, and that CRP can also bind to IL-8 (the consequences were not so far studied). These data prompted us to study physical interactions between CRP, SAP and IL-8, and to evaluate their role in the process of transendothelial migration of PMNs.

## MATERIALS AND METHODS

### *Human C-reactive Protein, Serum Amyloid P Component, Fibronectin and IL-8*

CRP was isolated from ascites fluid of carcinoma patients, purified and assessed as described elsewhere [4]. In SDS-PAGE it showed single band of approximately 22 kDa corresponding to CRP subunits. CRP was kept at 4°C in citrate buffer (pH 6.2) with 0.01% sodium azide and before use was dialyzed against 1000-fold volume of PBS at 4°C during 18 h and sterile filtered. SAP was purified as described by others with slight modifications [5]. A polyclonal antiserum against SAP was obtained in rabbits by three injections (once a week) of 50, 140 and 120  $\mu$ g of SAP in Freund's complete adjuvant. An IgG fraction was prepared by ion exchange chromatography [6]. FN was a purified, electrophoretically homogeneous protein isolated from pooled human plasma as described [7]; it was kindly provided by Dr. I.V. Voronkina (Institute of Cytology, St. Petersburg, Russia). IL-8 was an electrophoretically pure recombinant human cytokine produced in the Institute of High Pure Chemicals (St. Petersburg, Russia) by expression in *E. coli*. It was concentrated to 320  $\mu$ g/ml and kept at -20°C until use. IL-8 and rabbit neutralizing polyclonal antibodies against IL-8 were kindly provided by Prof. A.S. Simbirtsev.

### *Protein Binding Assay*

Binding of IL-8 to immobilized CRP was studied by ELISA. Titertech plates was coated with CRP (10  $\mu$ g/ml) by overnight incubation at 4°C and blocked with 1% bovine serum albumin (BSA, Sigma, USA) containing 0.05% Tween-20 (Sigma, USA). IL-8 (0.5  $\mu$ g/ml) was allowed to bind to immobilized CRP for 1 h at 37°C. Then the wells were incubated with polyclonal antiserum against IL-8 at 2-fold dilutions for 1 h at 37°C. All washings were made with the same BSA-Tween solution. Bound anti-IL-8 IgG was detected with a peroxidase-conjugated goat anti-rabbit IgG (Pasteur Institute of Epidemiology and Microbiology, St. Petersburg, Russia) and o-phenylenediamine/hydrogen peroxide substrate in phosphate-citrate buffer (pH 5.0). Adsorbability was measured at

492 nm with a plate reader (Spectra-III, Austria). Binding of IL-8 to immobilized SAP was studied under similar conditions.

#### *The Binding Assay in Solution*

CRP (400 µg/ml) and IL-8 (300 µg/ml) were mixed, incubated for 1 h at 37°C, layered on 15-30% sucrose gradient in 20 mM Tris-HCl (pH 7.4) and centrifuged at 100 000 g using Beckman L7-55 centrifuge (rotor SW50.1) for 19 h at 4°C. The tube content was separated into 14 fractions (350 µl each), and protein in each aliquot was precipitated with 10% TCA at 0°C during 1 h. Precipitates were sedimented, washed and dried on air and analyzed by SDS-PAGE [8].

#### *Isolation of PMNs*

PMNs were isolated from heparinized peripheral blood of healthy donors by the following procedures: centrifugation on Ficoll-Verografin gradient (1.077 g/cm<sup>3</sup>), collecting the bottom mononuclear-depleted fraction and its resedimentation in 9% polyvinyl alcohol (55 kDa) solution for 30-45 min at 37°C, and removal of erythrocytes with 0.824% ammonium chloride for 20 min at 4°C. After each incubation the cells were washed 3 times with PBS. The final cell population consisted of approximately 95% of neutrophils. Neutrophils were suspended at concentration of 10<sup>6</sup> cell/ml in Hanks' balanced salt solution (**HBSS**) containing 10% fetal calf serum (**FCS**) and used in migration and adhesion studies.

#### *Flow Cytometry Analysis*

For flow cytometry, PMNs were obtained from blood in a simplified way, including spontaneous sedimentation of erythrocyte and lysis of the rest erythrocytes. PMNs (1.0x10<sup>6</sup> cell/ml) were stimulated by CRP, SAP, IL-8 or their mixtures for 30 min at 37°C and washed twice with HBSS containing 0.1% sodium azide. Then the cells were incubated with mAb against CD11b/CD18 (Medbiospecter, Moscow, Russia) or mAb against fibronectin receptor (Sigma, USA) for 30 min at 4°C, washed and incubated in FITC-conjugated goat anti-mouse IgG (Medbiospecter, Moscow, Russia) under the same conditions. After wash-

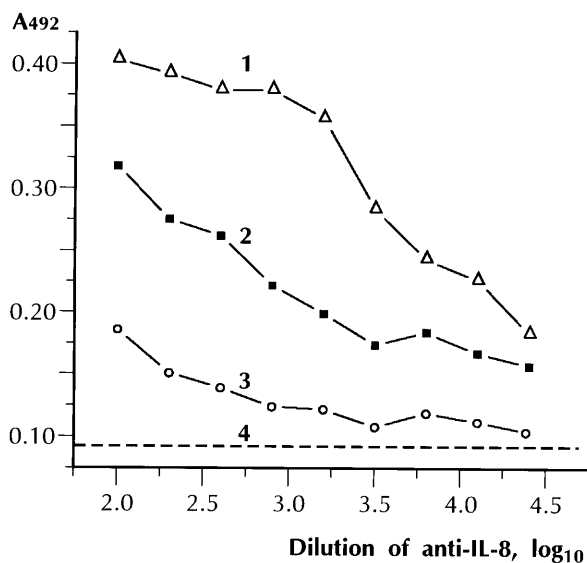
ing, PMNs were resuspended in 1% paraformaldehyde, and single-color immunofluorescent staining was analyzed by EPICS-XL cytofluorimeter (Beckman-Coulter, USA). Negative controls were obtained by omitting monoclonal antibodies. Data from 5 000 events were sampled. Antibody binding was determined as mean fluorescence intensity and quantity of PMNs after gating for neutrophils by their characteristic forward and side scatter properties.

#### *Evaluation of Neutrophil Adhesion*

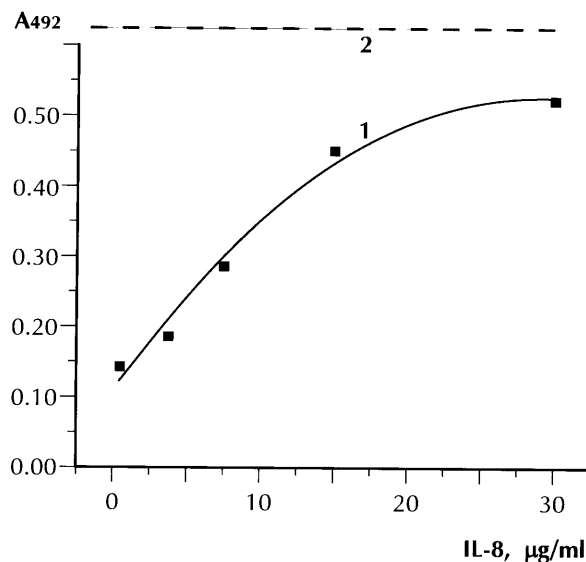
PMN adhesion was studied using FN-coated plastic wells. FN (5 µg/ml) was allowed to immobilize in 96-well flat-bottom Linbro plates for 2 h at 18°C. After washing with PBS the wells were filled with PMNs (7x10<sup>4</sup> cells/well), and CRP, SAP, IL-8 or their mixtures (for concentrations, see below) were added. The plates were incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, and unattached PMNs were removed by three washings with RPMI 1640 medium containing 10% FCS. Attached cells were dried, fixed with methanol and stained with 0.1% crystal violet. An excess of dye was removed by washing. Cell-bound dye was extracted by 0.1% SDS (0.2 ml/well) for 3 h at 37°C, and the adsorbability of solution was measured in fresh plate at 620 nm.

#### *Evaluation of Neutrophil Migration*

PMN migration was assessed by a method of cell migration under agarose layer [9]. The assay was performed in 90-mm Petri dishes precoated with human FN (5 µg/ml, 2 h/37°C). Melted 1% agarose (Sigma, USA) in MEM culture medium (Gibco, USA) supplemented with 10% FCS was poured onto dishes to give a thin layer, and 3-mm wells were cut in the gel after its solidification. The wells were filled with 20 µl of PMNs (1x10<sup>6</sup> cell/ml in the same medium). CRP, SAP, IL-8 or their mixtures in 20-µl volumes were added to empty wells situated 12 mm away from the wells with PMNs. PBS or human serum albumin were used as controls. After incubation for 18-24 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, the dishes were fixed with methanol and stained



**Figure 1.** Interaction between IL-8 and immobilized C-reactive protein. (1) - immobilized IL-8 + anti-IL-8; (2) - immobilized CRP + free IL-8 + anti-IL-8; (3) - immobilized CRP + anti-IL-8; (4) - immobilized BSA + anti-IL-8.



**Figure 2.** Interaction between IL-8 and immobilized serum amyloid P component. (1) - immobilized SAP + free IL-8 + anti-IL-8 (1:100); (2) - immobilized IL-8 + anti-IL-8 (1:100). Background adsorbability (non-specific binding of anti-IL-8 polyclonal antibody to immobilized SAP in the absence of IL-8) is subtracted.

with 0.1% amido black 10B (Reanal, Hungary). Then the agarose was carefully removed and the areas of cell migration were measured under MBS-10 microscope (LOMO, St. Petersburg, Russia) at 16-fold magnification. Results are presented as chemotaxis index (**CI**) - a ratio of migration distance toward experimental protein (expressed in ocular micrometer units) to migration distance toward control well.

#### Evaluation of Anti-Streptolysin O Activity of CRP

Influence of IL-8 binding on biologic activity of CRP was assessed with a method of CRP-mediated inhibition of hemolytic effect of streptolysin O (**SLO**) [10, 11]. Briefly, fifty microliters of two-fold dilutions of recombinant human IL-8 in PBS containing CRP (30 µg/ml) were incubated with 30 µl (10 hemolytic units) of SLO (Institute of Vaccines and Sera, St. Petersburg, Russia) for 30 min at 37°C, and 20 µl of 1% suspension of human erythrocytes in PBS was added. After additional incubation for 30 min at 37°C, the degree of hemolysis was registered

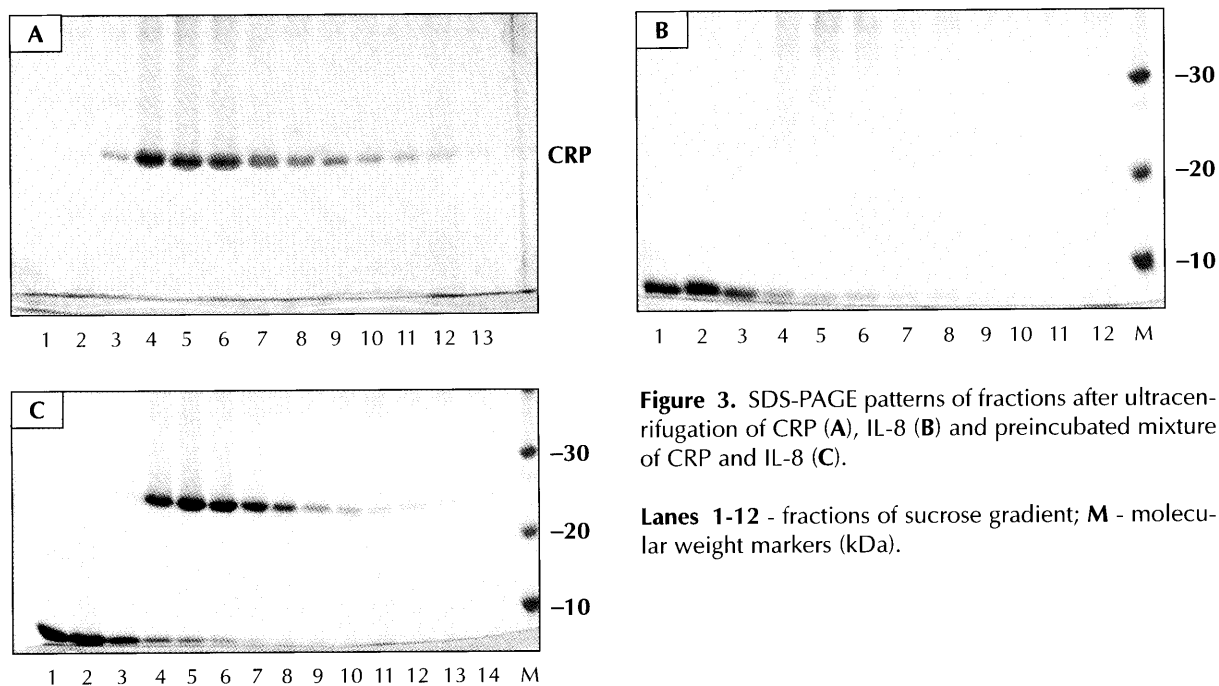
by measuring the adsorbability of the supernatants at 405 nm.

## RESULTS

### Binding of IL-8 to Immobilized Acute Phase Proteins (CRP and SAP) as Assessed by ELISA

Immobilized CRP bound to IL-8 at physiological ionic strength (**Figure 1**). A quantity of CRP at immobilization was 75 pM (10 µg/ml) and the quantity of added IL-8 was 568 pM, so the molar ratio of CRP to IL-8 was approximately 1:7.5, based on MW of 125 000 for CRP and 8 800 for IL-8. Interleukin-8 binding was detected with polyclonal antiserum against IL-8 which was prior tested for cross-reactivity with CRP by incubating twelve 2-fold dilutions (starting from 1:100) in CRP-coated wells. The dilutions showing no cross reaction were chosen for use.

Since IL-8 might require calcium ions during binding to CRP like other ligands of CRP, we compared the binding of IL-8 to CRP in the presence of Ca<sup>2+</sup> ions and under calcium-free conditions. The binding of IL-8 to immobilized CRP appeared to be independent of calcium because



**Figure 3.** SDS-PAGE patterns of fractions after ultracentrifugation of CRP (A), IL-8 (B) and preincubated mixture of CRP and IL-8 (C).

**Lanes 1-12** - fractions of sucrose gradient; **M** - molecular weight markers (kDa).

the addition of 2 mM  $\text{Ca}^{2+}$  ions did not influence the interaction of CRP with IL-8 (data not shown). When the wells precoated with IL-8 were allowed to bind free CRP, the binding was not clearly registered. Thus, immobilized CRP evidently binds to IL-8 whereas immobilized IL-8 doesn't bind to free CRP. This indicates that the primary immobilization of CRP is the necessary requirement for interaction of these proteins.

SAP was also studied for ability to bind to IL-8. SAP was adsorbed onto microplate wells in a quantity of 43.7 pM and allowed to bind to IL-8 (113.6 pM). The molar SAP/IL-8 ratio of added proteins was approximately 2:5. The results presented in **Figure 2** show that immobilized SAP behaves similarly to CRP and can also bind to IL-8. Binding occurred at physiological ionic strength, in PBS not supplemented with calcium (Fig. 2).

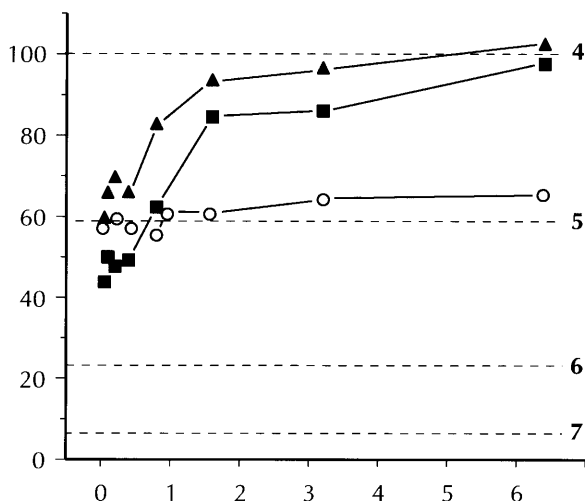
#### *Interaction Between CRP and IL-8 in Solution as Assessed by Ultracentrifugation and SDS-PAGE*

Results of ultracentrifugation of preincubated mixture of CRP and IL-8 in isokinetic gradient of sucrose are shown in **Figure 3**. Fig. 3a demonstrates gradient protein profile after centrifugation

of CRP. CRP was distributed over fractions 4 to 11 and was negligible in fractions 1, 2, and 3. After ultracentrifugation of IL-8, the bulk quantity of cytokine was revealed in the first three fractions (Fig. 3b) with traces of protein in fractions 4, 5, and 6. Thus, the distribution of free CRP and IL-8 under isokinetic sucrose gradient centrifugation was quite different. The distribution of the proteins after mixed preincubation did not change: as earlier, the peak of IL-8 was found in fractions 1-3 and CRP in fractions 4-11 (Fig. 3c). If the interaction occurred in solution, the protein profile would have change after gradient centrifugation, since CRP-IL-8 complex would be of a larger size and would have a higher constant of sedimentation. These data suggest that CRP and IL-8 do not interact in solution.

#### *Interaction of IL-8 with CRP as Assessed by the Change of Anti-Streptolysin O activity of CRP*

Binding of CRP to SLO can be inhibited by certain ligands of CRP (most effectively by poly-L-lysine) [11]. Similar decrease of anti-SLO activity of CRP has been shown to be induced by a 30-min preincubation of CRP with human recombinant IL-8 (**Figure 4**). IL-8 *per se* had no



**Figure 4.** Decrease of antitoxin activity of CRP after preincubation with IL-8

(1) - SLO + CRP (3.2 µg/ml) + IL-8; (2) - SLO + CRP (1.6 µg/ml) + IL-8; (3) - SLO + CRP (1.6 µg/ml) + IL-2; (4) - SLO (2.5 hemolytic units); (5) - SLO + CRP (1.6 µg/ml); (6) - SLO + CRP (3.2 µg/ml); (7) - PBS.

X axis: concentration of IL-8 (µg/ml) and IL-2 ( $10^{-3}$  U/ml);  
Y axis: hemolysis (%).

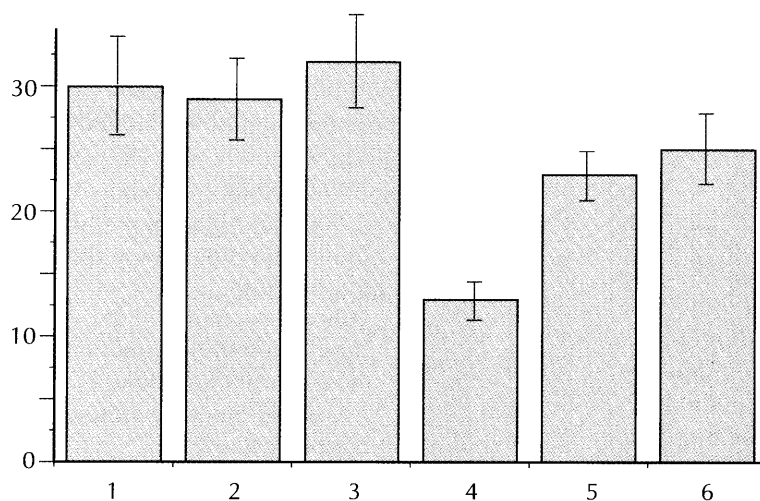
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influence on hemolytic activity of SLO at a wide range of doses, and another recombinant human cytokine, IL-2, didn't affect anti-SLO activity of CRP (Fig. 4). An apparent ability of IL-8 to affect one of the activities of CRP supports the ELISA data that IL-8 binds to CRP. Amounts of IL-8 reducing antitoxin effect of 1.6 and 3.3 µg/ml CRP by 50% were determined by plots (Fig. 4) fitted with polynoms of 2 and 3 order. Four determinations gave the mean of IL-8 equal to  $8.93 \pm 1.74 \times 10^{-8}$  M. The molar concentrations of CRP were  $1.28 \times 10^{-8}$  M (1.6 µg/ml) and  $2.64 \times 10^{-8}$  M (3.3 µg/ml). So, the molar ratios of CRP to IL-8 at 50% inhibition point were 1:6.3-1:8.3 for 1.6 µg/ml of CRP and 1:2.9-1:3.5 for

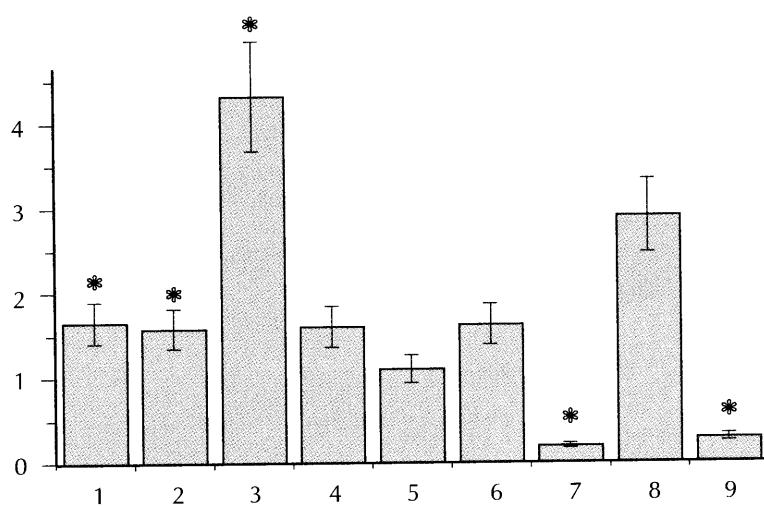
3.3 µg/ml of CRP. These ratios are close to those obtained with ELISA.

#### *Influence of Acute Phase Proteins and IL-8 on Adhesion of PMNs to Fibronectin*

The data presented in **Figure 5** show the influence of CRP, SAP, IL-8 and their preincubated mixtures on PMN adhesion to FN (control adhesion of PMNs to FN was taken as the zero level). IL-8 (0.5 µg/ml) lowered PMN adhesion to FN by 30% (column 1,  $P < 0.05$ ). CRP (1 µg/ml) + IL-8 (0.5 µg/ml) as well as SAP + IL-8 (at the same concentrations) decreased the adhesion of PMNs to FN by 29% and 32%, respectively (Fig. 5, columns 2 and 3,  $P < 0.01$ ). When the concentra-



**Figure 5.** Effects of IL-8, CRP and SAP on human neutrophil adhesion to fibronectin-coated substrate (percentage of control). (1) - IL-8; (2) - IL-8 + CRP (1 µg/ml); (3) - IL-8 + SAP (1 µg/ml); (4) - IL-8 + CRP (10 µg/ml); (5) - IL-8 + SAP (10 µg/ml); (6) - IL-8 + CRP (1 µg/ml) + SAP (1 µg/ml). IL-8 was used at concentration of 0.5 µg/ml. The numbers of observations are: 15 - for 1, 2, 3 and 6, and 8 - for 4 and 5. The following differences - 1 vs. 4, 1 vs. 5, and 4 vs. 5 - are statistically significant at  $P \leq 0.05$  (by White criterion)



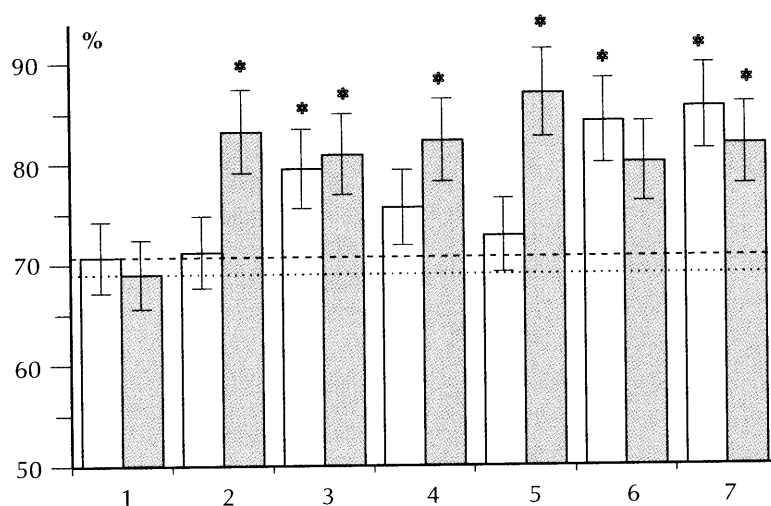
**Figure 6.** Effects of CRP, SAP and their mixture on PMN migration on fibronectin-coated substrate (CI). (1) - IL-8; (2) - CRP; (3) - SAP; (4) - IL-8 + CRP (expected migration); (5) - IL-8 + CRP (actual migration); (6) - IL-8 + SAP (expected migration); (7) - IL-8 + SAP (actual migration); (8) - SAP + CRP (expected migration); (9) - SAP + CRP (actual migration). IL-8 was used at 5  $\mu\text{g}/\text{ml}$ , CRP and SAP - at 10  $\mu\text{g}/\text{ml}$ . The following differences - 1, 2 and 3 vs. PBS; 5 vs. 4; 7 vs. 6, and 9 vs. 8 - are significantly different at  $P \leq 0.05$  (by Student's t criterion).

tions of acute phase proteins were increased to 10  $\mu\text{g}/\text{ml}$ , the degree of IL-8-dependent inhibition of adhesion decreased. Combinations of IL-8 with CRP (10  $\mu\text{g}/\text{ml}$ ) or with SAP (10  $\mu\text{g}/\text{ml}$ ) lowered the adhesion of PMNs only by 13% and 23%, respectively (columns 4 and 5, both effects are insignificant). Statistical analysis revealed significant differences between the effect of IL-8 and effects of several combinations of acute phase proteins with IL-8 (Fig. 5; compare columns 1 with 4, 1 with 5, and 1 with 6). These data suggest that binding of IL-8 to any pentraxin results in moderation of IL-8 influence on PMN adhesion. The effect of a three-protein mixture (Fig. 5; CRP + SAP + IL-8, column 6) was significantly

different from the effects of any of the two-protein mixtures (CRP + IL-8 or SAP + IL-8,  $P=0.01$ ). This indicates that CRP and SAP more readily bind to one another than to IL-8, and in the three-protein mixture a higher proportion of IL-8 molecules remains unbound by pentraxins.

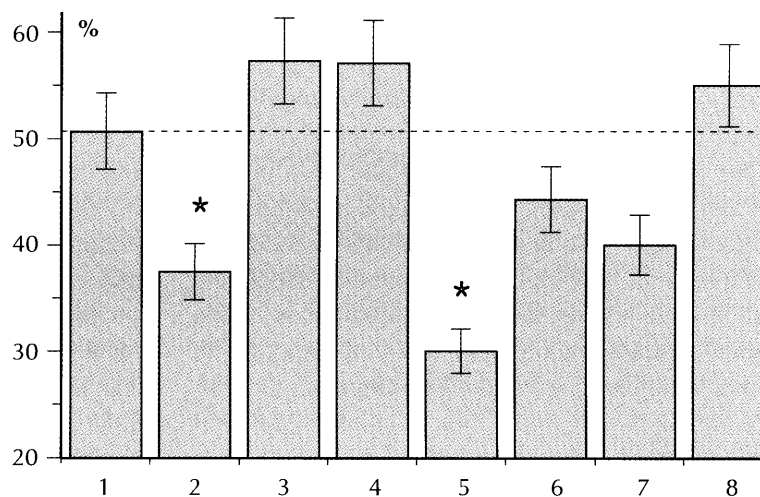
#### *Influence of Acute Phase Proteins and IL-8 on Migration of PMNs on FN-Coated Surface*

IL-8 stimulated directed movement of PMNs and enhanced chemotaxis index by 65% (CI=1.65, **Figure 6**). CRP and SAP also showed strong chemoattractive activities: their CIs were 1.58 and 4.32 (increase by 332%), respectively. To evaluate a joined effect of two (or three) pro-



**Figure 7.** Effect of CRP, SAP, IL-8 and their combinations on expression of CD11b(□)/CD18(■) by PMNs (percentage of CD11b/CD18-positive cells). (1) - PBS; (2) - CRP; (3) - SAP; (4) - CRP + SAP; (5) - IL-8; (6) - IL-8 + CRP; (7) - IL-8 + CRP + SAP. Dashed horizontal lines show control levels of CD11b<sup>+</sup> and CD18<sup>+</sup> cells obtained with PBS. Asterisks show statistically significant differences from corresponding controls at  $P < 0.05$  (by Student's t test or White criterion).

**Figure 8.** Effect of CRP, SAP, IL-8 and their combinations on expression of fibronectin receptors by PMNs (percentage of FNR-positive cells). (1) - PBS; (2) - CRP; (3) - SAP; (4) - CRP + SAP; (5) - IL-8; (6) - IL-8 + CRP; (7) - IL-8 + SAP; (8) - IL-8 + CRP + SAP. Dashed horizontal line shows control level of fibronectin receptor-positive cells obtained with PBS. Asterisks show statistically significant differences from corresponding controls at  $P < 0.05$  (by Student's t test or White criterion).



teins acting simultaneously, we used the «expected» CI which was calculated as an average of separate effects of each of the proteins, assuming that in a mixture each protein acted independently. The actual migration of PMNs to the wells with SAP + CRP mixture was very low, much less than expected (0.28 vs. 2.9). The pentraxins inhibited one another by 248% (Fig. 6, column 4). Similar result was obtained with PMN migration to the wells with CRP + IL-8 mixture. The actual CI for CRP + IL-8 mixture was 1.1, whereas the expected CI for CRP + IL-8 combination was 1.6 (the difference is statistically significant). The actual migration of PMNs to the wells with SAP + IL-8 was also low (CI=0.19) and was much less than expected (expected CI=1.62). Using chemotaxis as a model, we could demonstrate that interactions between pentraxins and IL-8 affect biological activity of the proteins. Low chemotactic activity of molecular pairs (of either CRP with SAP or of any pentraxin with IL-8) suggests that the binding between pentraxins or between pentraxins and IL-8 is associated with their inactivation.

#### *Influence of Acute Phase Proteins and IL-8 on Expression of CD11b, CD18 and FNR by PMNs*

The average amounts of CD11<sup>+</sup> and CD18<sup>+</sup> cells among intact PMNs were  $70.7 \pm 7.8$  and  $69.0 \pm 7.8\%$ , respectively. Numbers of positive cells correlated with mean fluorescence intensity of PMNs

(receptor density on cell surface). CRP didn't affect the level of CD11b expression, while SAP significantly increased both the number of CD11b-positive PMNs and the density of receptors on cell surface (**Figure 7**,  $P < 0.05$ ). The mixture of these proteins (CRP + SAP) didn't change the level of CD11b compared to control one, which confirms that SAP was inactivated by another pentraxin. However, any of pentraxins as well as their mixture elicited elevation of CD18 receptor expression by PMNs (Fig. 7). IL-8 elevated the number of CD18<sup>+</sup> cells like CRP. The mixture of IL-8 with CRP lost this ability, while the mixture of IL-8 with both CRP + SAP showed it again (Fig. 7). This indicates that CRP and IL-8 can bind and neutralize one another, but do not form complex in the presence of SAP presumably because of higher affinity of CRP to SAP than to IL-8. At the same time, the mixtures IL-8 + CRP and IL-8 + CRP + SAP elicited elevation of the number of CD11b-positive cells.

About 50% of intact PMNs expressed receptors for FN ( $\alpha_5\beta_1$  integrins, **Figure 8**). CRP and IL-8 lowered the number of FNR<sup>+</sup> cells (to 37.5% and 30%, respectively,  $P < 0.05$ ), whereas SAP slightly and insignificantly elevated it (to 57.3%,  $P > 0.05$ ) (Fig. 8). The effect of CRP + SAP on the expression of FNR was different from that of CRP: SAP blocked activity of CRP and abolished inhibitory effect of the latter on FNR expression (Fig. 8,  $P < 0.05$ ). Under the action of CRP + IL-8

or SAP + IL-8, the magnitude of CRP-associated or IL-8-associated inhibition of the number of FNR-positive PMNs was also less profound (Fig. 8,  $P < 0.05$ ). The pronounced cross-neutralization of CRP and IL-8 was observed, if the third protein (SAP) was added to their mixture: real effect of CRP + SAP + IL-8 combination (55% of FNR<sup>+</sup> PMNs) was very low; it was significantly different from the expected effect calculated for these three proteins (41.6%,  $P < 0.05$ ) and didn't differ from background effect of PBS (Fig. 8).

## DISCUSSION

Interactions between major acute phase inflammatory factors are one of the most intriguing regulatory mechanisms of inflammation. Our group as well as some foreign researchers have shown that there is a direct interaction between two acute phase proteins, CRP and SAP [12, 13]. When these proteins bind one another, they lose some of their activities. Other experimental data suggested that CRP also binds to IL-8 [13, 14]. During the present study the interaction between CRP and IL-8 was confirmed by ELISA. Immobilized CRP interacted with IL-8 at physiological ionic strength after being adsorbed onto plastic surface. No apparent binding of IL-8 was seen when CRP and IL-8 were both free in solution. The results of ultracentrifugation and SDS-PAGE of the CRP and IL-8 mixture showed that the proteins most probably did not interact in solution. This suggests that circulating CRP and IL-8 do not bind each other during acute phase of inflammation. This suggestion is indirectly supported by the absence of reports about circulating complexes containing CRP and IL-8. Conformational changes of CRP molecule known to occur under immobilization [12] could lead to the opening of hidden sites responsible for IL-8 binding. Nevertheless, the data presented herein show that preincubation of CRP with IL-8 in solution apparently changed their effect on PMN functions. This indicates that binding of IL-8 by CRP in this case should be preceded by prior binding of CRP to a certain substrate, for example to cell membranes or to serum lipoproteins, fibronectin, *etc.*

SAP is highly homologous to CRP, and both pentraxins have similar spatial structures. Immobilized SAP may interact with IL-8 under the same conditions as CRP, though IL-8 binding by SAP seemed to be some weaker.

Pro-inflammatory cytokine IL-8 is known to stimulate a number of activities of neutrophils including their adhesion to plastic surfaces. At the same time IL-8 has been shown to inhibit PMN adhesion to polycarbonium membrane [15]. Our data indicating that high concentration of IL-8 inhibits PMN adhesion to immobilized FN suggest that IL-8 can act as anti-inflammatory factor as well.

High concentrations of pentraxins (10  $\mu\text{g}/\text{ml}$ ) neutralized IL-8, CRP being more effective in inhibiting IL-8 than SAP. The effect of three-protein combination (CRP + SAP + IL-8) was different from the effect of two-protein mixture (SAP + IL-8). This indicates that CRP more readily interacts with SAP than with IL-8.

Using synthetic peptides and monoclonal antibodies to defined regions of CRP molecule, two binding sites for SAP have been identified: the first matched to peptide 134-148 (ILGQEQDSFGGNFEG), the second - to peptide 191-206 (YEYVQGEVFTKPQLWP) [12]. Interaction of CRP with SAP is independent on lectin specificities of CRP, since a number of sugars didn't interfere with CRP-SAP binding. In SAP molecule, a site for binding CRP was matched to peptide 144-199 of carboxyterminal part of SAP [12]. A competition between IL-8 and SAP for binding with CRP is worthy of special study.

One of the mechanisms of inhibitory effect of IL-8 on PMN adhesion to FN was elucidated by the data on expression of FNR on PMN surface (Fig. 8). Acting separately, IL-8 and CRP lowered the number of FNR-positive PMNs, but no significant effect on FNR expression was seen under their joined action. CRP and IL-8 neutralized one another. Thus, the pathways of regulatory action of CRP + IL-8 combination on the expression of different integrins (FNR vs. CD11/CD18) seem to be different. In contrast to CRP and IL-8, SAP elevated the expression of FNR by PMNs. Nevertheless, the addition of IL-8 to SAP diminished this effect of SAP too. CRP

could not weaken the activity of SAP, however simultaneous addition of CRP and IL-8 to SAP inactivated SAP evidently resulting in apparent recover of FNR expression on PMN surface. Although the mechanisms of influence of various combinations of proteins remain obscure, it can be suggested that CRP and IL-8 interact with different sites of SAP.

The results clearly have shown that pentraxins SAP and CRP easily neutralize chemotactic activity of IL-8 and cross-neutralize one another. IL-8, CRP and SAP, all chemoattractive for PMNs in single use, demonstrated loss of their chemoattractant activities in any combination. Our data indicate that the acute phase proteins influence PMN adhesion and migration, thus implicating CRP and SAP in regulation of transendothelial migration of PMN.

IL-8 initiates the second phase of transendothelial migration, namely, the phase of tight adhesion. This process is associated with the increasing of CD11/CD18 expression and avidity. In our experiments, SAP was similar to IL-8 in activating expression of two subunits of CD11/CD18 integrin complex. So, SAP is thought to be able to accelerate the phase of tight adhesion of PMNs like IL-8.

It is interesting that in activating CD11b expression SAP showed no decrease of its activity when mixed with CRP which was in distinct contrast to the results pertinent to chemotaxis and adhesion of PMNs. Moreover, marked and significant stimulatory effect on the expression of CD11b was elicited by the mixture of CRP with IL-8 although neither CRP nor IL-8 showed stimulatory potential after single use. Similarly, CRP and SAP did not interfere one another in the activation of CD18 expression by PMNs. This suggests that the sites of CRP, SAP and IL-8 involved in the triggering of integrin expression are different from those required for chemotactic activity of these proteins and/or for induction of PMN adhesion.

In summary it may be concluded that CRP and IL-8 can promote the movement of PMNs along the extracellular matrix by attracting PMNs and diminishing FNR expression on their surface. The role of SAP and three-component system CRP +

SAP + IL-8 in regulation of PMN migration along the extracellular matrix should be clarified in further investigations.

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