Characterization of the Chemotactic Activity of Casein for Neutrophil Leucocytes and Macrophages[1]

The acid-precipitable casein fraction of the milk proteins exerts a strong chemotactic attraction in vitro for neutrophil leucocytes and for macrophages[2,3]. Nevertheless, there have been no published attempts to characterize the fractions of casein involved of the physicochemical requirements for activity. There has indeed been a degree of doubt whether the activity was due to casein itself or to a contaminant, since bacteria, some of which produce chemotactic factors, may contaminate milk. An examination of this activity is reported here, from which it may be concluded that chemotactic activity is a property of the casein molecules themselves, and is modified by procedures which typically modify the physicochemical properties of casein.

The crude acid-precipitated fraction of whole bovine milk was used as a source of casein. This was obtained commercially as casein 'Hammarsten' or casein 'alkalilöslich' (Merck, Darmstadt, Germany). Satisfactory casein preparations were usually insoluble in neutral solutions but could be dissolved by raising the pH to 12. They then stayed in solution when brought back carefully to neutrality. Purified α-casein was obtained from Sigma Chemical Corp., USA.

Whole casein was fractionated by: a) Gel filtration on Sephadex G-100 (Pharmacia, Uppsala, Sweden) using distilled water as eluant. Previous workers fractionating casein on Sephadex have used 6 *M* urea as eluant[4] but the same threepeak pattern on G-100 was obtained using water (Figure 1): b) Calcium chloride precipitation[5]. A modification of this procedure was used to fractionate Hammarsten casein. Three fractions were obtained: 1. a *K*- and β-casein rich supernatant after addition of 0.25 *M* $CaCl_2$; 2. an α-casein rich fraction precipitated with 0.25 *M* $CaCl_2$ and re-precipitated by 0.07 *M* $CaCl_2$; 3. a β-casein-rich fraction precipitated with 0.25 *M* $CaCl_2$ but soluble in 0.07 *M* $CaCl_2$.

The chemotactic activity of casein was tested after digestion by incubation at 37 °C for 10 min to 1 h with the following proteolytic enzymes: crystalline trypsin, crystalline chymotrypsin, plasmin (plasminogen/streptokinase) and thrombin (200 μg enzyme to 5 mg casein).

Chemotactic assay was carried out as described previously[6,7]. The cells used were either human peripheral blood neutrophils or guinea-pig macrophages obtained from the peritoneal cavity 4 days after i.p. injection of paraffin oil. Neutrophil chemotaxis was measured using filters of 3 μm pore size (Millipore, Bedford, Mass. USA) and macrophage chemotaxis using filters of 8 μm pore size (Sartorius, Göttingen, Germany).

All preparations of whole casein and of purified α-casein in which the casein was in solution were strongly chemotactically active for both human neutrophil leucocytes and for guinea-pig (and rabbit) peritoneal exudate macrophages (Table I). Casein preparations which were insoluble or poorly soluble in Gey's solution were poorly chemotactic. Solubility, rather than the source or method of preparation of the casein, appeared to be the most important determinant of chemotactic activity. The chemotactic activity of casein preparations was also affected by Ca^{++} concentration. In the absence of Ca^{++}, cell motility was poor. On increasing the $CaCl_2$ concentration to 0.06 *M* a sharp increase in the chemotactic response of the cells to casein was seen. At Ca^{++} concentrations higher than this, there was a gradual drop in chemotactic activity of casein, associated with a drop in solubility of the casein preparation, which formed micelles as Ca^{++} concentration was raised[8].

An unusual feature of the chemotactic activity of casein was that it could be abolished or grossly diminished by addition of the casein preparation to whole milk (Table I). Milk was itself only slightly chemotactic. The possibility was considered that milk contains an inhibitor of chemotaxis. However, no such inhibitor could be isolated on fractionation of milk and it seems more likely that the lack of chemotactic activity of casein in milk is due to the fact that such casein is present in the form of non-diffusible high molecular weight micelles, since milk has a Ca^{++} ion concentration much higher than other biological fluids.

Table I shows that the chemotactic activity of casein was grossly reduced by prior hydrolysis of the protein with trypsin, chymotrypsin and plasmin but was un-

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Table I. Effect of incubation with proteases or of readdition to milk on the chemotactive activity of casein for human neutrophil leucocytes

Chemotactic factor (5 mg per ml)	Nutrient medium	Enzyme added	Chemotactic activity (Neutrophils per H.P. field on lower surface of filter; mean of 3 tests)
Casein 'Hammarsten'	Gey's	None	197
Casein 'Hammarsten'	Gey's	Trypsin	15
Casein 'Hammarsten'	Gey's	Chymotrypsin	12
Casein 'Hammarsten'	Gey's	Plasmin	47
Casein 'Hammarsten'	Gey's	Thrombin	212
Casein 'Hammarsten'	Whole milk (bovine)	None	20
None	Whole milk (bovine)	None	11
α-casein	Gey's	None	134
None	Gey's	None	0

Table II. Chemotactive activity of casein fractions prepared by precipitation with calcium chloride

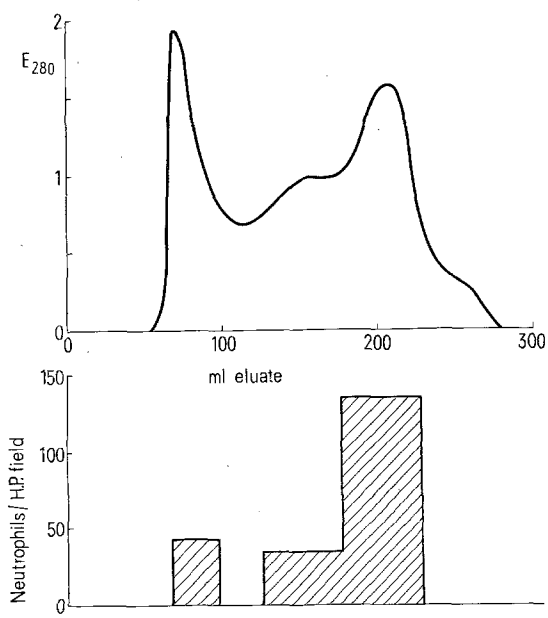
Fraction (at 10 mg per ml)	Cell counts on lower surface of filter	
	Neutrophils per H.P. field (mean of 6 filters)	Mactophages per H.P. field (mean of 6 filters)
Whole casein	112	107
Fraction I (K and β-casein-rich) 0.25M $CaCl_2$ supernatant	13	13
Fraction II (α-caseinrich) 0.07M $CaCl_2$ precipitate	51	24
Fraction III (β-caseinrich) 0.07M $CaCl_2$ supernatant	30	not tested

affected by incubation with thrombin. This pattern of inhibition of chemotaxis parallels exactly the susceptibility of casein to digestion by this group of proteolytic enzymes[9]. Acid hydrolysates of casein had no chemotactic activity.

The chemotactic activity of Sephadex G-100 fractions of casein is shown in the Figure. The major activity was associated with the most slowly eluted of 3 protein peaks. The approximate molecular weight of this protein was 20,000. Thus, the casein was probably in monomeric form in this peak since the molecular weight of the monomeric forms of several casein fractions has been calculated to lie between 20,000 and 30,000[10].

Table II shows the chemotactic activity of calcium chloride precipitated casein fractions. Major activity was seen in the α- and β-casein-rich fractions and the K-casein rich fraction was much less active.

The results outlined here indicate that the chemotactic activity of casein is a property of the casein molecules themselves, and not due to impurities, for the following reasons: a) Chemotactic activity is inhibited by enzymes, e.g. trypsin, which destroy the casein molecule but not by enzymes, e.g. thrombin, which do not. b) Chemotactic activity is associated with a discrete peak on gel filtration of casein which corresponds with the monomeric form of the protein. c) The chemotactic activity of casein shows a dependence on Ca^{++} concentration which parallels its change from soluble to micellar form as Ca^{++} ions are added. Insoluble preparations of casein are not chemotactic; d) purified α-casein or α-casein-rich fractions are chemotactic.



Sephadex G-100 chromatography of whole casein in distilled water showing distribution of protein peaks (above) and chemotactic activity (below). The 3 peaks had respective calculated molecular weights of 1. > 100,000; 2. 35,000 (approx.) and 3. 20,000 (approx.).

There are two characteristics of casein which could give clues to its chemotactic properties. Firstly, it is a phosphoprotein and, secondly, α-casein[11] and β-casein[12] are both highly unusual inasmuch as they have a random coil conformation at neutral pH. If phosphate groups in casein are important in chemotactic recognition, it would be unusual since other chemotactically active proteins contain no phosphorus. The random coil conformation of casein is seen in other globular proteins only after denaturation. If proteins such as serum albumin, which are normally tightly folded, are denatured at acid pH or by reduction-alkylation, their chemotactic activity increases[13]. Thus an unfolded protein structure may assist chemotactic recognition of proteins by phagocytic cells. However, in its physiological environment, i.e. milk, casein forms micelles and is thus protected from attack by phagocytes.

Résumé. L'activité chimiotactique de la caséine envers les neutrophiles et les macrophages se manifeste dans ses fractions soluble de poids moléculaire bas. Cette activité est détruite par l'action des enzymes protéolytiques et après la formation des micelles. Les α- et β-caséines sont les plus actives. Le manque de structure tertiaire dans la molécule de caséine peut jouer un rôle dans son activité chimiotactique.

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