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Interaction of Casein with Human Polymorphonuclear Cells

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Since the first demonstration of the chemoattracting effect of casein (1) it has been widely used in the investigation of the chemotaxis of various mammalian cells (2–10). Whole casein represents a heterogenous population of molecules of about 400,000 Da which can be separated into α (α_s and χ), β, and γ fractions by electrophoresis. Chemotaxis can be induced by α-, χ-, or β-casein (11–12); however, little is known about the mechanism by which casein induces migration.

The interpretation of the biological effect is rendered difficult by insufficient knowledge of the physicochemical properties of casein. Even the concentration of casein in solution is difficult to define because it is determined by the equilibrium of small soluble complexes and large micelles (13–14) which is influenced by the pH, temperature, and ion composition of the medium (15–20). The formation of casein micelle at neutral pH is mainly due to α-casein (75% of total casein). Further, β-casein (22%), which exists as a monomer at 4°C, shows a strong tendency to associate into polymers at higher temperature (21–22).

Hydrophobic interactions between the receptors and the chemoattractant has been considered to be important in the induction of chemotaxis by Wilkinson (11,23–26). Casein is rich in nonpolar groups which are exposed on the surface on the molecule even in aqueous medium (25) and they may easily bind to the membrane lipid of cells. Moreover casein attaches to cellulose nitrate filters or glass. A filter coated with casein induces chemotaxis, in the absence of fluid-phase protein (27).

This paper deals with the binding of ^{125}I-labeled casein to blood polymorphonuclear (PMN) leukocytes. Though a complex interaction with cells was observed, our data do not support the mediation of chemotactic action of whole casein through specific surface receptors.

MATERIALS AND METHODS

Purification of casein. Commercial casein may be contaminated with lipids derived from epithelial cells (28) which may induce chemotaxis; therefore casein was extracted with ethanol, chloroform/methanol, and then with hexane (5). Thereafter low molecular weight protein fragments were removed by precipitation

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using 3% TCA. Finally, casein was dissolved in NaOH and adjusted to pH 7.4 by HCl.

Preparation of cells. PMNs were obtained from healthy adult donors by dextran sedimentation, according to Ushijima and Nakao (29). Contaminating red cells in the PMN fraction were removed by hypotonic lysis. The PMN (88–94% neutrophils) were suspended in Gey's solution. Cell viability as measured by trypan blue due exclusion was ≥ 97%. PMNs were finally suspended in Gey's solution (1–4 × 10^6 cell/ml). Lymphocytes and erythrocytes were prepared by the procedure of Boyum (30).

Radioiodination. Purified casein was labeled with ^{125}I by the method of McConahey and Dixon (31).

Chemotaxis assay. The morphologic method of Zigmond and Hirsch was used to assess PMN chemotaxis (32). A single 3.0-μm cellulose nitrate filter (Sartorius, Göttingen) was used and the chemotactic chambers were incubated for 45 min at 37°C. The migration front was determined by measuring the farthest distance traveled by two cells per high-power field with a standard microscope micrometer.

Binding assay. The binding of ^{125}I-casein was assayed as described by Harvath and Leonard (33). Cells were suspended in Gey's solution containing 0.1% bovine serum albumin, or without albumin. After incubation, the suspensions were filtered onto Whatman glass fiber filters (GF/C). The filters were washed with 40 ml cold Gey's solution, and were placed in scintillation vials with 10 ml scintillation fluid, and counted in a Beckman LS spectrometer.

RESULTS

Chemotaxis induced by native and iodinated casein. In order to show that the biological activity of whole casein was not changed by ^{125}I-labeling, a sample was subjected to the iodination procedure using higher amounts of unlabeled NaI. The treatment did not influence the chemotactic activity of casein as shown in Fig. 1. In view of the dose–response curve (Fig. 1A) in the experiments casein was applied at concentrations of 0.1 to 10 mg/ml. The numbers of PMN cells migrating to successive 10-μm levels in a 1-mm^2 area of the filter were counted.

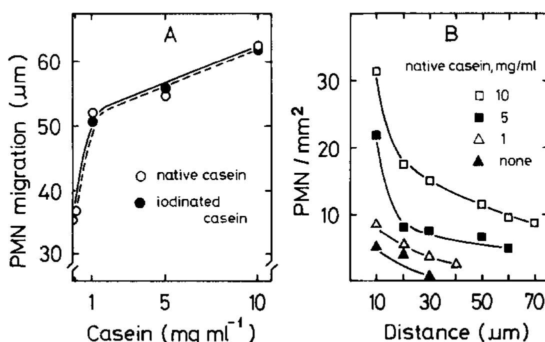

Fig. 1. The effect of native, or iodinated casein, on the chemotaxis of human PMN (A) and on PMN distribution within the membrane filter (B).

The shape of the resulting curves (Fig. 1B) indicated that the cell population was homogenous as regards its chemotactic responsiveness.

Attachment of casein to the cellulose nitrate filter and its diffusion into the upper compartment of the migration chamber. Since casein was shown to attach to the filter (27) we investigated whether it can enter the upper chamber containing PMN. Casein was applied into the lower compartment at different concentrations and we measured the amount of casein attached to the filter and its concentration in the upper compartment after various intervals. Depending on the dose, 100–300 μg casein attached rapidly to the filter and its amount did not increase significantly after 10 min (Fig. 2A). The concentration of casein in the upper compartment increased in a time-dependent manner at high doses of casein. At lower doses the upper concentration hardly increased after 10 min (Fig. 2B).

Characteristics of the binding of ^{125}I-casein to PMN cells. The kinetics of the binding of ^{125}I-casein to PMN at 24°C was determined in the absence and presence of albumin (0.1 mg/ml). Albumin is known to enhance the binding of formyl peptide to the leukocytes (34) and to inhibit membrane proteases (35). In our experiment albumin decreased the binding of ^{125}I-casein by 50% (Table 1). Albumin attaches aspecifically to the filter or glass, as well as to cells inducing chemokinesis (12, 36–38) and it may cover the surface sites for casein.

An inhibition of membrane proteases by albumin doses not seem to be important in this respect since phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) did not decrease the binding of ^{125}I-casein (see below). Therefore albumin was omitted from the media in the following experiments, since the chemotactic effect of casein manifested itself in the absence of albumin, as well.

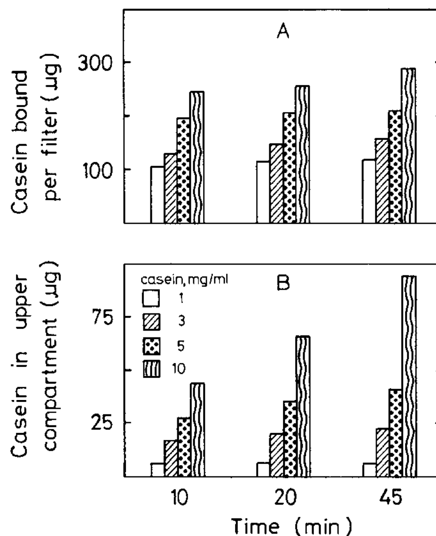

FIG. 2. Binding of casein to membrane filter (A) and casein appearance in the upper compartment (B) as a function of time. Under the conditions of the chemotactic assay the protein content of the upper solution and of the membrane filter were determined at the indicated time points by the method of Lowry *et al.* (52). The filters were previously washed with Grey's solution three times.

TABLE 1
Effect of Bovine Serum Albumin on the Binding of ^{125}I-Casein in to Human PMN

Time (min)	^{125}I-casein bound/10^6 cells (cpm) Control	BSA added
3	750	460
10	1330	570
20	2000	920
45	2700	1180

Note. Cells (4 × 10^6/ml) were incubated with ^{125}I-casein (6 μg/ml) in the absence or in the presence of BSA (100 μg/ml).

The binding of ^{125}I-casein to PMN was investigated at 37, 24, and 4°C in order to decrease the internalization and proteolysis of casein. The time course of the binding (Fig. 3) was similar at 24 and 37°C. Cell-bound radioactivity increased in proportion to time for 30 min; maximum binding was observed after about 45 min. At 4°C the binding increased at a lower rate for 180 min. The following experiments were performed at 24°C.

Figure 4 shows that the addition of 10- and 100-fold excess of unlabeled casein did not displace the cell-associated molecules, moreover the binding proceeded at lower rates.

To determine if a specific saturable receptor for casein existed on PMN, ^{125}I-casein binding was examined in the presence of various amounts of unlabeled casein (Fig. 5). Since binding was observed even at a 2500-fold excess, the increasing uptake indicated nonspecific association.

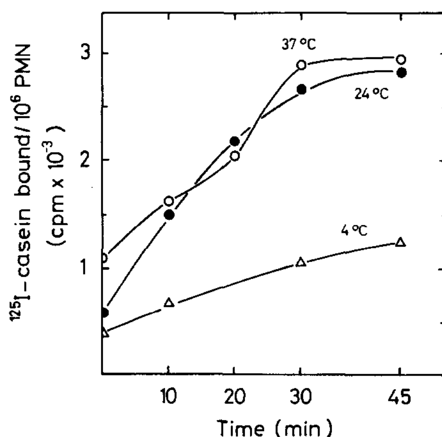

FIG. 3. Binding of ^{125}I-casein to human PMN at various temperatures. PMNs (4 × 10^6/ml) were incubated with ^{125}I-casein (0.5 μg/ml) at 4 and 37°C.

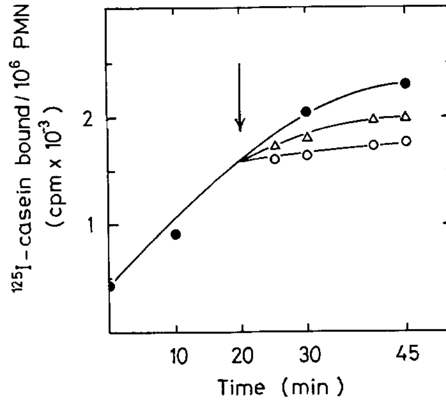

FIG. 4. Effect of unlabeled casein on ^{125}I-casein binding to PMN. Cells (4×10^6/ml) were incubated with ^{125}I-casein (6 μg/ml) at 25°C. A large excess of unlabeled casein (△) 100 μg/ml, or (○) 1000 μg/ml, was added after 20 min of incubation.

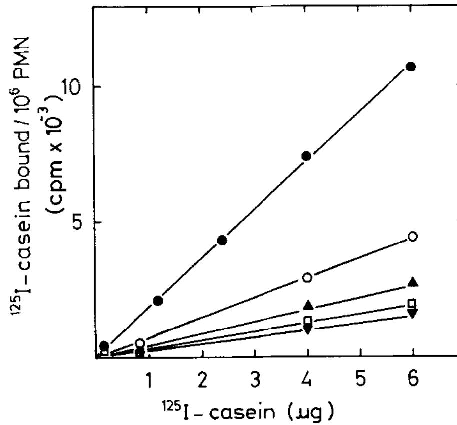

FIG. 5. Binding of ^{125}I-casein to PMN as a function of increasing concentration of labeled casein. Various amounts of ^{125}I-casein (0.16–6 μg/ml) were incubated with PMN (4×10^6 cells/ml) for 30 min in the absence (●) or presence (○, 25-, ▲, 100-, □, 750-, and ▼, 2500-fold) of excess unlabeled casein.

Dissociation of cell-bound ^{125}I-casein was investigated at 4, 24, and 37°C. PMN cells were incubated with ^{125}I-casein for 45 min at 24°C then washed and transferred into a casein-free medium. After incubation at different temperatures for 60 min the cell-bound radioactivity was determined (Fig. 6). The release was faster at 37°C, at which temperature only 38% of the initial radioactivity remained cell-bound.

The binding of casein was investigated after pretreatment of cells with 50 μg/ml trypsin and 0.25% *n*-butanol for 20 min, which disturbed the structure of cell membrane (34,39,40). Further, the effects of pretreatment with 10^{-2} M NaN_3 [blocking endocytosis (41)], 10^{-4} M indomethacin [inhibitor of cyclooxygenase (42)], and 2×10^{-3} M PMSF (43), were also examined. None of these agents

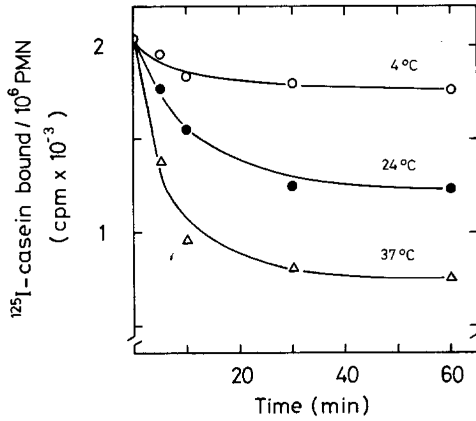

FIG. 6. Release of PMN-bound ^{125}I-casein at various temperatures. Cells (4 × 10^{6}/ml) were incubated with labeled casein (1 μg/ml) at 24°C for 45 min. After washing, PMNs were incubated in casein-free Gey's solution at the indicated temperature.

altered the attachment of casein. As it was observed with other chemoattractants these treatments inhibited chemotaxis (11,39,40,44–46).

The significance of hydrophobic forces in the binding was investigated by pretreatment of PMN with polar and nonpolar amino acids, lysine, tryptophan, and leucine. A concentration-dependent inhibition of ^{125}I-casein binding was exerted by leucine only (Fig. 7). The inhibition was also observed when leucine was added to the incubation medium 20 min after the addition of ^{125}I-casein.

Attachment of ^{125}I-casein to lymphocytes and erythrocytes. Peripherial blood cells were isolated and incubated with ^{125}I-casein as shown in Fig. 8. Both lymphocytes and erythrocytes bound casein though at a lower rate than did PMN. Further, the attachment of casein increased steadily for 45 min.

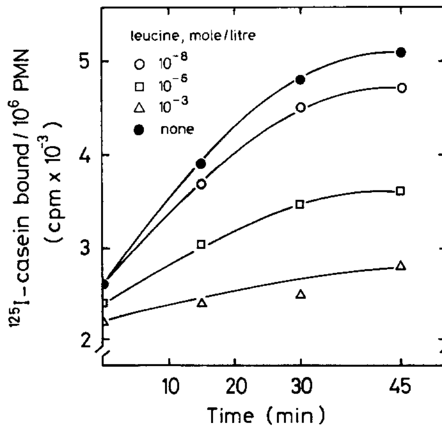

FIG. 7. Effect of leucine on the binding of ^{125}I-casein to PMN. Cells (4 × 10^{6}/ml) were incubated with ^{125}I-casein (6 μg/ml) in the presence of the indicated concentrations of leucine at 24°C.

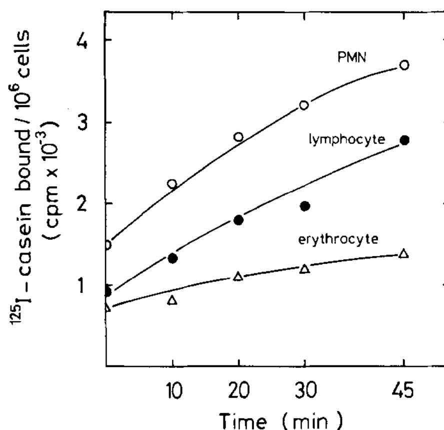

FIG. 8. Binding of ^{125}I-casein to human erythrocytes, lymphocytes, and PMN. Cells (2 × 10^6/ml) were incubated with ^{125}I-casein (6 μg/ml) at 24°C. Cell-bound radioactivity was measured as described for PMN under Materials and Methods.

DISCUSSION

Our investigations on the attachment of native casein to PMN cells indicate that casein (at chemotactic doses) is bound aspecifically. This was shown by the following findings: (i) ^{125}I-Casein attachment occurred even at 4°C, and increased with time (Fig. 3). (ii) Bound ^{125}I-casein was not displaced by large amounts of unlabeled casein (Fig. 4). (iii) No saturation was found at a 2500-fold excess of unlabeled casein (15 mg/ml, Fig. 5). (iv) The attachment was not influenced either by modification of the cell surface by trypsin or *n*-butanol, or by PMSF and indomethacin. (v) The attachment of casein to PMN was inhibited by a hydrophobic amino acid, leucine (Fig. 7). Nevertheless, the association between ^{125}I-casein and PMN was considerably stable at 4°C and also at 37°C (Fig. 6).

The attachment to various cell types correlated with their chemotactic response to casein; the binding was minimal in the case of the nonmigrating erythrocytes, whereas PMN and lymphocytes bound more casein (Fig. 8). However, [^{3}H]formyl peptide has been shown to bind to both migrating and nonmigrating subpopulations of human PMN (47). On the other hand erythtrocytes, thrombocytes, and lymphocytes do not bind formyl peptide (48–49).

Specific binding of formyl peptides (50,41), cleavage product of the fifth complement component (48) and leukotriene B_4 (51), has been demonstrated on PMN surface. Binding sites were also described for isolated α-casein fraction on peritoneal macrophages (47) which, however, were saturated far below the chemotactic concentration of α-casein (11,12). In our experiments the attachment of whole casein to PMN was investigated at concentrations which induce chemotaxis. It can be calculated from the data of Fig. 5 that a single PMN cell can bind 4 × 10^7 casein molecules (at 15 mg/ml casein concentration, 24°C in 30 min).

Our results suggest that the induction of chemotaxis may be due to the formation of casein–membrane lipid complexes, stabilized by hydrophobic forces. It can

be assumed that not individual casein molecules but micellar complexes of casein interact with the PMN membrane.

SUMMARY

Attachment of ^{125}I-casein to PMN cells was investigated. Iodination did not decrease the chemotactic effect of casein. ^{125}I-casein binding was increasing toward a maximum reached at about 45 min at 24, and 37°C. At 4°C the binding was proportional to time for 45 min. No saturation was achieved even at 15 mg/ml casein. About 40% of casein remained attached to PMN in a casein-free medium after 60 min, at 37°C. Pretreatment of the cells with trypsin or butanol, or the presence of indomethacin, azide, and PMSF did not affect the binding of casein. The hydrophobic amino acid, leucine counteracted the attachment of casein.

Our data show that at chemotactic doses casein is bound aspecifically to cell membranes by hydrophobic forces. The induction of chemotaxis may be due to micellar casein–membrane lipid complexes.

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