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SPECIFIC HIGH-AFFINITY BINDING SITES FOR A SYNTHETIC GLIADIN HEPTAPEPTIDE ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Summary

The synthetic peptide containing residues 43-49 of α-gliadin, the major protein component of gluten, has previously been shown to inhibit the production of lymphokine activities by mononuclear leukocytes. We now demonstrate using radiolabeled α-gliadin(43-49) that human peripheral blood lymphocytes express approximately 20,000-25,000 surface receptors for this peptide, with a dissociation constant (K_D) of 20 nM. In addition, binding is inhibited by naloxone and an enkephalin analog, thus confirming the functional correlate which demonstrates inhibition by these agents of α-gliadin(43-49) functional effects. Furthermore, B-lymphocytes bind specifically a greater amount of [^{125}I]α-gliadin(43-49) than T-lymphocytes. The lymphocyte α-gliadin(43-49) receptor may play an important role in mediating the immunological response to α-gliadin.

The mechanism by which gluten intolerance is related to the manifestation of coeliac disease is still unclear. However, experimental results support the hypothesis that lymphocyte-mediated immunity to gliadin, the major protein component of gluten, may be involved in the pathogenesis of the disease (1-3). Lymphocytes within the mucosa of the small intestine (2) and in the peripheral blood of patients with coeliac disease have been shown to produce elevated amounts of the lymphokine leukocyte migration inhibition factor (LIF) (4-7). The observed functional interactions between the sensitized lymphocytes and gliadin, or gliadin substituent peptides, have suggested the presence of specific gliadin receptors on the surfaces of these cells (8,9).

The observation that the opiate antagonist naloxone inhibits the effect of α-gliadin on lymphocytes from patients with coeliac disease has led Horváth *et al.* (7) to propose that the effect of α-gliadin and its substituent fragments may be mediated by opioid-like receptors on lymphocytes. Recently, the two homologous peptides corresponding to residues 43-47 and 43-49 of α-gliadin (10) have been shown to have the same effects as the intact molecule on the stimulation of LIF production by lymphocytes from patients with coeliac disease (11).

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We report here that a radiolabeled derivative of one of these peptides, α-gliadin(43-49) designated gliadorphin, with the amino acid sequence Tyr-Pro-Gln-Pro-Gln-Pro-Phe binds specifically to normal human peripheral blood lymphocytes (HPBL), and that the binding is saturable and linear with cell number.

Methods

Peptide radioiodination: The gliadorphin peptide was iodinated by the method of Hunter and Greenwood (12). To 0.1 ml of 0.2 M phosphate buffer (pH 7.5), 20 μg (~20 nmoles) of the peptide and 2 mCi sodium iodide [^{125}I] (Amersham) were added. Then 20 μl of chloramine-T (0.5 mg/ml) were added and after 20 sec 20 μl of sodium metabisulfite (1 mg/ml) were added. The reaction mixture was applied to a Sephadex G-10 column (20 ml total volume) previously equilibrated with 0.2 M phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin. The first 3 ml of the radioactivity peak were used for the binding studies. The specific activity of the radiolabeled peptide was estimated to be 100 Ci/mmole.

Isolation of human peripheral blood lymphocytes: Mixed leukocytes from heparin-anticoagulated blood of normal subjects were centrifuged on Ficoll-Hypaque cushions to resolve mononuclear leukocytes from polymorphonuclear leukocytes. The mononuclear leukocytes at the buffer and Ficoll-Hypaque interface were washed twice and resuspended at a density of 1 x 10^8/ml of RPMI with 25 mM HEPES (pH 7.4) and 0.5% bovine serum albumin (w:v). Monocytes were removed by incubating 1 x 10^7/ml mononuclear leukocytes in the above tissue culture medium in the presence of 10% (v:v) human AB serum and incubated in 75 cm^2 plastic tissue culture flasks for 1 hr at 37°C in 5% CO_2:95% air. The contents of the flask were decanted, and the nonadherent lymphocytes (approximately 70% T-lymphocytes and 30% B-lymphocytes) were resuspended at a density of 1 x 10^8/ml of tissue culture media. The purity of the lymphocytes was greater than 95%, with less than 3% monocytes detected by a nonspecific esterase stain. Viability of the mixed lymphocytes was always greater than 95%, as determined by the exclusion of trypan blue dye (13).

In order to separate T- from B-lymphocytes, the lymphocyte mixture was incubated with neuraminidase-treated fresh sheep erythrocytes to achieve rosetting of the T-lymphocytes (13). The mixtures were centrifuged on Ficoll-Hypaque cushions to separate the T-lymphocyte rosettes from the less dense non-rosetting B-lymphocytes. The erythrocytes were lysed by hypotonic exposure and the T-lymphocytes were washed and resuspended in tissue culture media. The purity of the T-lymphocytes was always greater than 95%.

Measurement of the binding of [^{125}I]gliadorphin to lymphocytes: In each experiment, a concentration of [^{125}I]gliadorphin ranging from 0.1 to 2.0 x 10^6 cpm without and with different concentrations of gliadorphin, α-gliadin, naloxone, and an enkephalin analog, (D-Met^2,Pro^5)-enkephalinamide (14) was incubated with duplicate suspensions of 2 x 10^7 lymphocytes in a final volume of 0.2 ml for 45 min at 4°C. The amount of bound radioactivity was determined by sedimenting the lymphocytes in each suspension through a 0.1 ml layer of phthalate oils (13) in a 0.4 ml conical polypropylene tube that was centrifuged for 1 min at 12,000 x g in a Beckman microfuge. The tip of the polypropylene tube containing the lymphocyte pellet was cut off with a razor blade and counted in a γ-counter. Non-specific binding of [^{125}I]gliadorphin was the amount of radioactivity bound in the presence of 10^{-5}M gliadorphin.

Results

Determination of the conditions for steady-state binding of [^{125}I]-gliadorphin by human peripheral blood lymphocytes: Different concentrations of human peripheral blood lymphocytes (HPBL) were incubated with [^{125}I]-gliadorphin and the total and non-specific binding of the peptide were determined. The specific binding of [^{125}I]gliadorphin increased linearly with the concentration of HPBL and correlated significantly ($r = 0.87$) over the range 1×10^6 to 1×10^7 HPBL (Fig. 1).

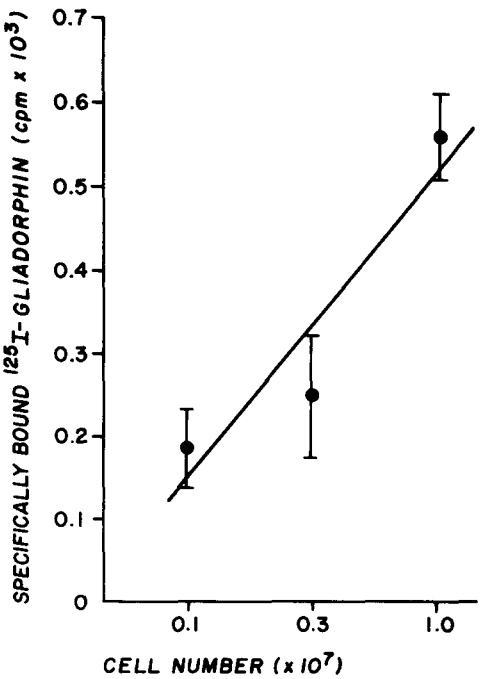

FIG. 1. Binding of [^{125}I]gliadorphin as a function of the concentration of HPBL. The number of HPBL indicated on the abscissa were incubated with 1×10^6 cpm of [^{125}I]gliadorphin in the presence and absence of 10^{-5}M unlabeled peptide for 45 min at 4°C. Specific binding was calculated by subtracting the nonspecific from the total binding. Specific binding of [^{125}I]gliadorphin showed a linear correlation ($r = 0.87$) with the concentration of HPBL over the range 1×10^6 to 1×10^7 cells. Each point and bracket are the mean of three measurements from two experiments, and standard deviations, respectively.

The time-course of specific binding of [^{125}I]gliadorphin by HPBL was examined by incubating duplicate suspensions of 1×10^7 HPBL with 1×10^6 cpm of [^{125}I]gliadorphin for 5 to 90 min at 4°C. The specific binding of the peptide by HPBL increased rapidly and reached a steady state after approximately 45 to 50 min at 4°C (Fig. 2).

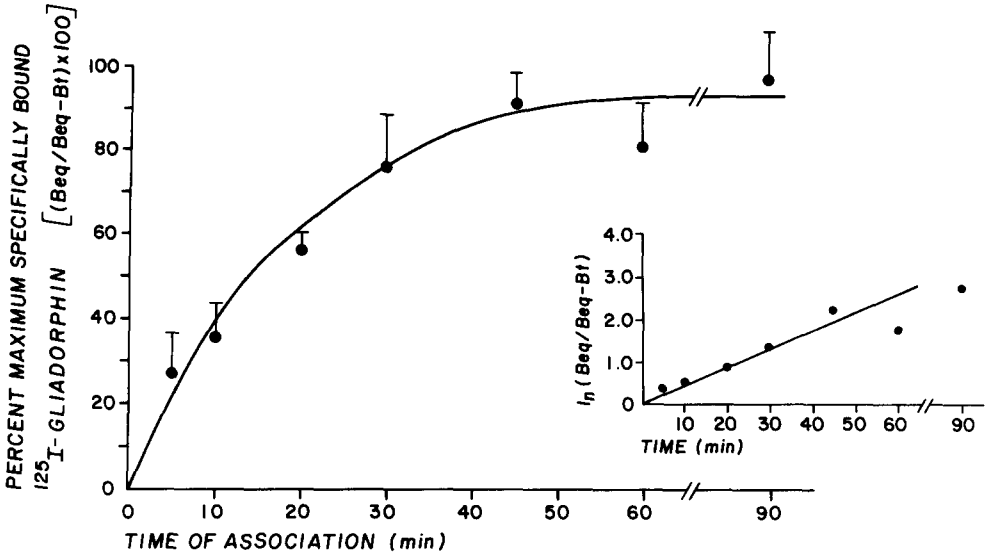

FIG. 2. The time-course of binding of [^{125}I]gliadorphin to HPBL. Duplicate suspensions of 1 x 10^7 HPBL were incubated with 1 x 10^6 cpm of [^{125}I]gliadorphin with and without 10^{-5}M unlabeled peptide, and the amount of specifically bound [^{125}I]gliadorphin was measured at intervals from 5 to 90 min at 4°C. **Inset**: Transformation of data from association curve into semi-logarithmic form, demonstrating pseudo-first order kinetics. Each point and bracket represent the mean ± S.D. of duplicate determinations from three separate experiments.

Steady-state characteristics and structural determinants of the specific binding of [^{125}I]gliadorphin to HPBL: Saturability of binding to 1 x 10^7 HPBL under steady state conditions was demonstrated in the concentration range of 0.1 to 2.0 x 10^6 cpm of [^{125}I]gliadorphin in the presence and absence of 10^{-5}M unlabeled peptide (Fig. 3A).

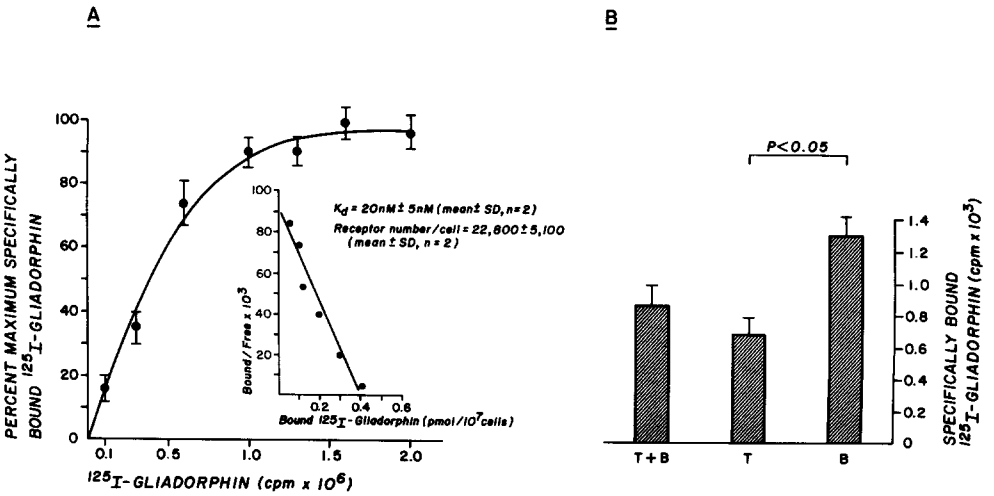

Fig. 3. Specific binding of [^{125}I]gliadorphin to HPBL. (A). Duplicate suspensions of 1 x 10^7 HPBL were incubated with [^{125}I]gliadorphin at concentrations from 0.1 to 2.0 x 10^6 cpm (specific activity 100 Ci/mmole) with and without 10^{-5}M unlabeled peptide at 4°C for 45 min. Specific binding was measured as described in the Methods. Each point and bracket represent the mean ± S.D. from two separate experiments. Inset: Scatchard analysis of a representative experiment of the specific binding of 1 x 10^6 cpm of [^{125}I]-gliadorphin to 1 x 10^7 HPBL. **(B).** Duplicate suspensions of 1 x 10^7 HPBL, T-lymphocytes, or B-lymphocytes were incubated with 1 x 10^6 cpm of [^{125}I]gliadorphin and the specific binding measured as above. The results are the mean ± S.D. of three experiments.

Under these conditions, the level of non-specific binding to HPBL was 35% ± 10% (mean ± S.D.) of the total binding. An estimate of the K_D and gliadorphin receptor density derived from the Scatchard analysis of steady state binding of 1 x 10^6 cpm of [^{125}I]gliadorphin reveals a K_D of 20 ± 5

nM and a single class of 20,000-25,000 receptors per cell, respectively (Fig. 3A, inset). In order to determine the contribution to [^{125}I]gliadorphin binding by the different lymphocyte populations, mixed lymphocyte preparations were enriched for either T-lymphocytes or B-lymphocytes as described in the Methods. When the binding under steady state conditions of 1 x 10^6 cpm of [^{125}I]gliadorphin to 1 x 10^7 mixed lymphocytes, T-lymphocytes, or B-lymphocytes was compared, a significantly greater amount of the peptide bound specifically to B-lymphocytes than to T-lymphocytes ($p < 0.05$), suggesting that the former have a greater number of gliadorphin receptors/cell (Fig. 3).

The dependence of specific binding of [^{125}I]gliadorphin on different structural determinants was evaluated by examining the competitive effects on the steady state binding of the peptide by the native molecule α-gliadin, the opiate antagonist naloxone, and an enkephalin analog. α-gliadin, with a molecular weight of about 30,000, inhibited specific binding of [^{125}I]-gliadorphin by 50% at approximately 10- to 15-fold higher concentrations. Similarly, naloxone competitively inhibited specific binding by 50% at 10-fold higher concentrations. The enkephalin analog (14) was equally potent as the gliadin peptide in inhibiting binding (Fig. 4).

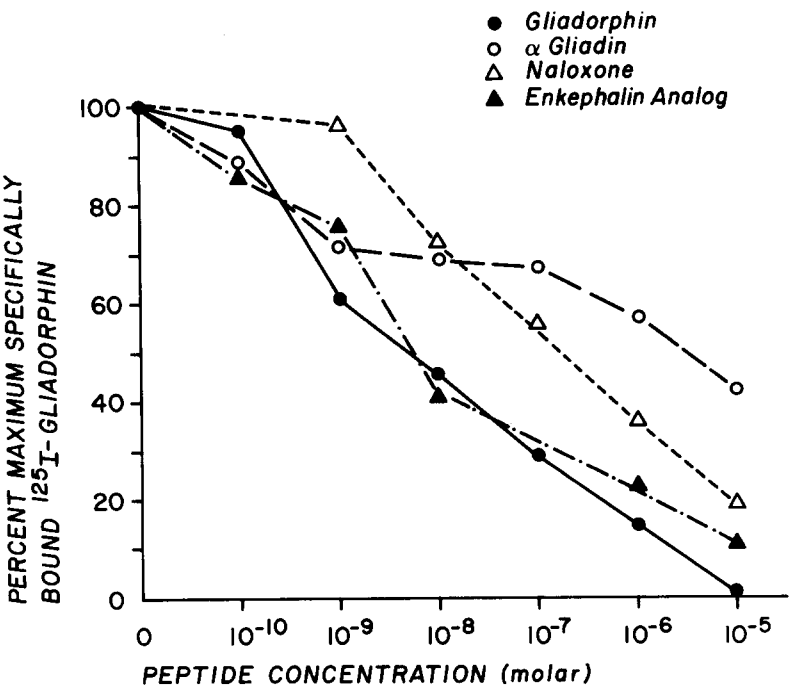

Fig. 4. Structural determinants of binding of [^{125}I]gliadorphin to HPBL. Each point and bracket depict the mean of two experiments done in duplicate ± SD of the percent specifically bound [^{125}I]gliadorphin in the presence of a range of concentrations of non-radioactive antagonists. • = gliadorphin; o = α-gliadin; △ = naloxone; ▲ = (D-Met^2,Pro^5)-enkephalinamide.

Discussion

We chose to synthesize and study α-gliadin(43-49), designated gliadorphin, because of its close structural homology to β-casomorphin Tyr-Pro-Phe-Pro-Gly-Pro-Ile, an exogenous opioid peptide (15). The gliadin peptide has been shown to have the same effect as the intact molecule on the stimulation of LIF production by lymphocytes from coeliac patients. It has also been shown that this effect could be blocked by naloxone (11). Since all the naloxone-reversible pharmacological effects are thought to be opioid-like ones, we have suggested that α-gliadin(43-49) might be structurally related to one of the exorphins formed by the enzymic digestion of dietary gluten (16).

Our present studies provide biochemical evidence that gliadorphin binds specifically to human peripheral blood lymphocytes. The binding of radiolabeled gliadorphin to HPBL is dependent on cell and peptide concentration (Fig. 1). The attainment of steady state binding of [^{125}I]gliadorphin to HPBL was rapid at 4°C (Fig. 2). Specific binding was saturable, with a K_D of about 20 nM and approximately 20,000-25,000 receptors/cell, with a greater number of receptors on B-lymphocytes compared with T-lymphocytes (Fig. 3).

The inhibition of the peptide binding by α-gliadin, naloxone, and a potent enkephalin analog (Fig. 4) suggests that this high-affinity binding of the peptide to HPBL may be related to the stimulatory effect of the peptide on LIF production by lymphocytes from coeliac patients (11). α-gliadin and gliadorphin, however, do not affect LIF production by lymphocytes from healthy individuals (4,5,7,11), which makes it necessary to perform direct binding studies in the future on lymphocytes from patients with coeliac disease. Furthermore, the pharmacological antagonism by naloxone of gliadorphin binding suggests the possibility that gliadorphin may be recognizing the lymphocyte opiate receptor, or that the gliadorphin receptor shares a significant degree of structural homology with the opiate receptors.

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