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ACTIVATION OF HUMAN T HELPER 1 AND DNAase EXPRESSION IN $CD4^+$ T CELLS INDUCED BY SHORT IMMUNOMODULATING PEPTIDES

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SUMMARY: Activation of human T helper 1 cells took place when lymphomononuclear cells from healthy donors were incubated in the presence of short synthetic peptides encompassing sequences present in extracellular matrix proteins. Active peptides conformed to a common structural pattern ("2-6-11 motif") [N.López-Moratalla et al., Biochem. Biophys. Acta (1994) 1221, 153-158] conferring immnunomodulating properties. The release of IL-2 and IFNγ , as well as LAK and NK-dependent cytotoxicity induced by these peptides,could be blocked by anti-HLA-DR antibody. Activated $CD4^+$ cells isolated from the mixed incubated population contained secretion granules with DNAase activity. These results suggest that these immunomodulating peptides presented by HLA-II play a key role in the differentiation of $CD4^+$ T cells towards a Th1 functional phenotype. © 1994 Academic Press, Inc.

$CD4^+$ lymphocytes are often classified on the basis of their pattern of lymphokine production. Two subclasses, Th1, which secretes IL-2 and IFNγ, and Th2, which secretes IL-4, have been described (1). It has been suggested that the Th1/Th2 ratio might be depending on the nature of the antigen presenting cell (APC); Th1 has been associated with activated B cells, whereas macrophages producing IL-1 with Th2 (2). Differences in interleukin production by Th cells mediate different immune responses. Macrophages are activated by Th1 cytokines increasing their lytic ability (3). Th2 cytokines coordinate the response of B cells (4). $CD4^+$ mediated cytotoxicity may involve multiple lytic mechanisms (5).

This report shows that immunomodulating peptides possessing a "2-6-11 motif" (6,7) and modelled after sequences present in extracellular matrix (ECM) proteins activated human Th1 clones and induced granule associated DNAase activity in $CD4^+$ cells.

Materials and methods

Cell source and preparations. Lymphomononuclear cells were isolated (from venous blood drawn from healthy donors) by centrifugation over Ficoll-Hypaque at 2 500 g for 15 minutes (8).

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When indicated, $CD4^+$ activated cells were separated after incubating the whole lymphomononuclear population with magnetizable polystyrene beads coated with a primary monoclonal antibody specific for the CD4 membrane antigen (Dynal, Oslo, Norway).

Cell culture conditions. Blood lymphomononuclear cells were cultured at $2x10^6$ per ml in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% of autologous serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) in humidified atmosphere with 5% CO_2 at 37°C.

Chemical synthesis of peptides. The synthesis of the various peptides was carried out by the solid phase method of Merrifield (9), with the Fmoc modification (10).

Cytokine determinations. IL-2, IL-4 and IFNγ were measured by the quantitative "sandwich" enzyme immunoassay technique with commercially available kits (Genzyme, Cambridge, MA). Whenever IL-2 had to be determined specific antibody against its receptor (anti-h-r-IL-2 Ab BT563, Biotest Pharma, Germany) was added to the incubation medium in order to avoid that binding of IL-2 to its soluble receptor could mask its release. Lymphomononuclear cells were placed in plates of 24 wells at a concentration of $2x10^6$ per ml and anti-h-rIL-2 mAb (5 µg/ml) was added. After seven days of incubation in the presence of the immunomodulating peptides, aliquots of 100 microliters of the supernatant of each well were used for IL-2 determinations (11). Monoclonal antibodies against HLA-DR were obtained as described in the literature (12) using L243 anti-DR hybridoma (American Type Culture Collection, Rockville, MD)

Cytotoxicity assay. The 4 h ^{51}Cr release cytolysis assay (13) was performed with K562 and Daudi cells as targets, essentially as previously described (6).

Extraction of granule proteins. The isolation of secretion granules was carried out using a Percoll density-gradient after disrupting the cells with a nitrogen bomb (14). Granule proteins were extracted following the procedure used by Podack et al. (15).

Detection of DNAase activity. The detection of DNAase activity in the protein extract was performed by its ability to degrade genomic DNA. Approximately 50 µg of the extracted granule proteins were incubated with 10 µg of protein free DNA at 37°C for 24 h in 100 microliters of a 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM Tris HCl buffer, pH 7.9. An aliquot of 50 microliters of the resulting mixture was applied to a 1% agarose gel, and subjected to electrophoresis at a voltage of 70 V in 1mM EDTA, 40 mM Tris HCl buffer, pH 7.5, containing 0.5 µg per ml of ethidium bromide. Genomic DNA was obtained from human blood as described in the literature (16).

Statistical analysis. Data were analyzed by the ANOVA and Fisher's PLsD tests, and a p value less than 0.05 was considered to be significant.

Results and discussion

Peptides modelled after sequences present in human ECM proteins were synthesized. All peptides contained 15 amino acids (14 of them encompassing sequences present in ECM proteins, to which an extra Val was added at the C-end for synthesis convenience) and a "2-6-11 motif" according to which a Pro was always present at position 6, Val, Leu, Ile, Ala, Lys at position 2, and Glu or Asp at position 11. The following peptides were used in this study: IKGLKPGVVYEGQLV (P1) and IISCHPVGTDEEPLV (P2) from fibronectin (17); VVRLVPENFRDFNTV (P3) and TKVSHPALLSDGKWV (P4) from laminin A (18); QALASPGSCLDEFRV (P5) from α1 collagen IV (19); Peptide NVLGAPKKLNESQAV (P6) not present in natural proteins, but having immunomodulating properties (6), and peptide ADAQQNKFNKDQQSV (Pc), lacking these properties and used as negative control (6). Peptides P1 through P6 were active in promoting the release of IL-2 and IFNγ, but not that of IL-4, when added to lymphomononuclear cells at a concentration of 30 µg per ml. Figure 1A shows

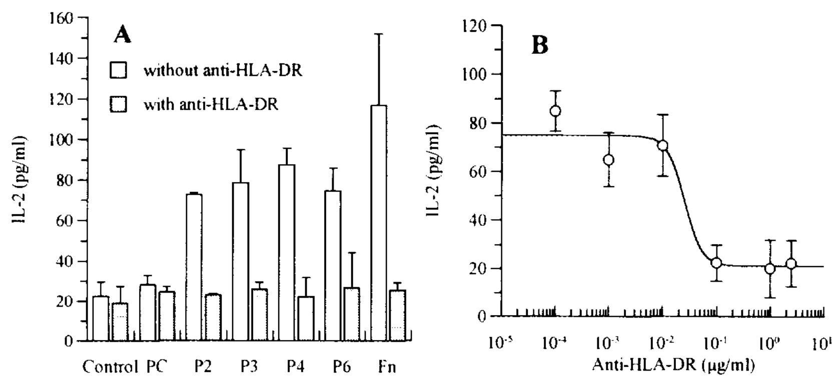

Figure 1. Release of IL-2 by human lymphomononuclear cells induced by immunomodulating peptides or fibronectin.
(A) Values represent the mean ± S.D of duplicates of three independent experiments using cells obtained from the blood of the same donor extracted at intervals of one week. The concentration of anti-HLA-DR was 2.5 μg per ml. The differences between cells treated with immunomodulators (peptides or fibronectin) and cells treated with control peptide or no peptide were statistically significant ($p<0.001$). Statistically significant differences ($p<0.001$) were also observed between values obtained in the presence and in the absence of anti-HLA-DR.
(B) Effect of anti-HLA-DR antibody concentration on IL-2 release by lymphomononuclear cells stimulated by active peptide P2. Values represent the mean ± S.D. (bars) of three independent experiments.

the values of IL-2 released to the medium after a 7-day incubation in the presence of active peptides or fibronectin as well as an inactive control peptide. The release of IL-2 elicited by active peptides or fibronectin was completely blocked, if anti-HLA-DR antibody had been present during the incubation. It may be seen that the values obtained with the inactive control peptide (Pc), in the presence or absence of anti-HLA-DR, were similar to those obtained with cells incubated under the same conditions, but in the absence of peptides. No significant differences between controls (inactive peptide or no peptide added) and immunomodulators in the presence of anti-HLA-DR. Figure 1B shows that a concentration of anti-HLA-DR antibody at least 0.1 μg per ml was necessary to block the release of IL-2 by active peptides. These results show that peptides presented by HLA-DR to $CD4^+$ cells induced a change towards a Th1 phenotype. Moreover, the activation of Th1 is further supported by the release of IFNγ (Figure 2), which was also blocked by anti HLA-DR antibody (Data not shown). Figure 1A shows also that the release of IL-2 induced by fibronectin was also blocked by anti-HLA-DR. This result could be explained if fibronectin undergoes cleavage leading to the formation of active peptides which could be presented by APC. It is worth noting that fibronectin contains within its sequence up to six times the "2-6-11" motif (7) separated by segments sensitive to proteases (20).

We had previously described that peptides with a "2-6-11 motif" induced LAK and NK dependent cytotoxicity (6,7). This cytotoxicity could also be blocked if anti-HLA-DR was present in the incubation (Data not shown). Cytokines released as a consequence of the presentation of immunomodulating peptides to Th1 could be mediating the induction of cytotoxicity.

In a different set of experiments we have isolated $CD4^+$ cells after the incubation of the mixed lymphomononuclear population in the presence of immunomodulating peptides. Protein extracts

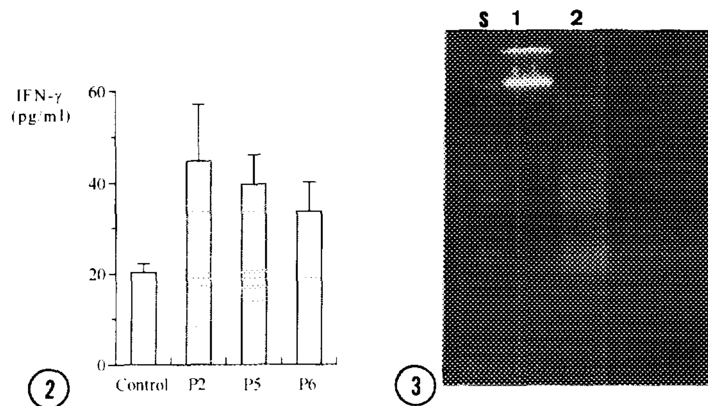

Figure 2. Release of IFNγ by human lymphomononuclear cells induced by immunomodulating peptides. Experimental conditions were the same as those of Figure 1. Values represent the mean ± S.D.of four experiments with cells obtained from different donors. Stastically significant differences ($p<0.05$) were observed between cells treated with immunomodulators (active peptides or fibronectin) and cells treated with inactive control peptide or no peptide.

Figure 3. Electrophoregram reflecting DNAase activity present in secretion granules of $CD4^+$ cells isolated from the mixed population of lymphomononuclear cells incubated in the presence of active peptide. Lane 1, protein free human genomic DNA. Lane 2, DNA fragmentation induced by peptide P6. Lane S, Standard DNA.

from secretion granules obtained from activated $CD4^+$ cells contained DNAase activity which could be detected by genomic DNA fragmentation (Figure 3). This observation is consistent with the existence of a mechanism of cytotoxicity involving secretion granules in Th1 cells. Grogg et al (21) have found that Th1 cells induce apoptosis of activated MHC class II cells. They also suggest that a transfer of nucleases cytotoxic lymphocytes to target cells might be taking place as part of this mechanism.

In conclusion, immunomodulating peptides possessing a "2-6-11 motif" (6,7) and modelled after sequences present in extracellular matrix (ECM) proteins activate human Th1 clones and induce granule associated DNAase activity in $CD4^+$ cells. The cytotoxic activity induced by these immunomodulating peptides (6,7) could be mediated by human $CD4^+$ cells in the case of tumor cell lines expressing HLA-II.

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