HETEROGENEITY OF MOUSE INTERLEUKINS

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1. Introduction

Supernatants from cultures of histo-incompatible spleen cells or spleen cells cultured in the presence of certain protein antigens or mitogens contain factors able to replace T cells during in vitro antibody synthesis [1,2]. Some of these show antigen specificity in their help, others are antigen-nonspecific. The name 'interleukin' was suggested [3] for the antigen-nonspecific factors. However, since these, in our studies, are biochemically very similar to an antigen-specific factor, we have used the same term for both groups of helper factors. Interleukin-1 [3] has a M_r of 12 000–18 000 and appears to be produced mainly by macrophages [4]. Interleukin-2 [3] is produced by T cells, has a M_r of 30 000–35 000 and is, in addition to its T cell-replacing activity, able to promote and maintain long-term cultures of T cells [3]. The biological and biochemical properties of the factors have been studied in several different laboratories, but their structural characterization have encountered great difficulties, partly due to the very small amounts of helper factor present in supernatants having high levels of biological activity, and partly due to the apparent molecular heterogeneity of the helper factors [1].

We have studied the heterogeneity of two differently induced helper factors. One was antigen-specific in its action and was induced with the protein antigen KLH, while the other one was antigen-nonspecific and induced with Con A. A series of conventional isolation procedures, such as ammonium sulfate precipitation, gel filtration and ion-exchange chromatography were employed to partially purify the factors. They were then further purified by reverse-phase HPLC and their app. M_r values determined by gel permeation HPLC in the presence of 6 M guanidine–HCl. It was found that even under denaturing conditions, it was not possible to obtain homogeneous factors. Biological activity was associated with material having M_r ~2000–60 000.

Abbreviations: app. M_r, apparent relative molecular mass; Con A, concanavalin A; DNP, 2,4-dinitrophenol; HPLC, high pressure liquid chromatography; KLH, keyhole limpet hemocyanin

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2. Materials and methods

For the production of Con A factor, spleen cells from C57B1/6 mice were cultured (5×10^6 cells/ml) in the presence of Con A (1 μg/ml) in serum-free Iscove's modified Dulbecco's medium (IMDM, Gibco [5]). Penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 2-mercaptoethanol (5×10^{-5} M) were added to the medium. After 24 h incubation, cells and cell-debris were removed by centrifugation and the supernatants were ammonium sulfate-precipitated (80% saturation). This material was further purified by gel filtration on Sephacryl S-200. The peak of biologically active factor was either analyzed directly by reverse phase HPLC (fig.1A) or further purified by ion-exchange chromatography (DEAE-cellulose) and gel filtration (polyacrylamide P60) before analysis by HPLC (fig.1B).

The KLH-specific helper factor was also produced from culture supernatant of C57B1/6 spleen cells, but in the absence of Con A and in the presence of KLH (0.1 μg/ml) (details in [6]). The helper factor was

purified by ammonium sulfate precipitation, ion-exchange chromatography, gel filtration and HPLC (fig.1C). The purification procedures for both factors have been described in detail (submitted).

HPLC [7] was done using RP8 or RP18 reverse phase columns packed with LiChrosorb (Brownlee Labs.). The HPLC gel permeation column was the I-125 protein column of Waters Associates.

The Con A factor activity was measured in a T cell replacing assay (stimulation of anti-sheep red blood cell antibody formation in cultures of nude spleen cells [8] and in a thymocyte proliferation assay (stimulation of thymidine incorporation by thymocytes [9]). The KLH-specific factor activity was measured in an in vitro antibody response assay in which spleen cells were incubated in the presence of DNP–KLH and the factor preparation to be assayed. After 4 days the number of plaque-forming cells were determined [6].

3. Results

In fig.1 are shown the HPLC elution profiles of the Con A-induced factor activity and the KLH-specific helper activity. The factor-containing samples were injected into reverse phase columns in 12 mM HCl and eluted with linear gradients of ethanol in the same acid. The approximate ethanol concentration which will elute a certain factor activity can be read on the abscissa. (A) and (B) both illustrate elution of Con A factor activity, but the factor material had been differently pre-treated in the two cases. After gel filtration on Sephacryl S-200 columns (see section 2) the peak of factor-activity was in one case (A) charged directly onto the reverse-phase HPLC column, but in another case (B) it was taken through additional steps of purification before analysis by HPLC. Appar-

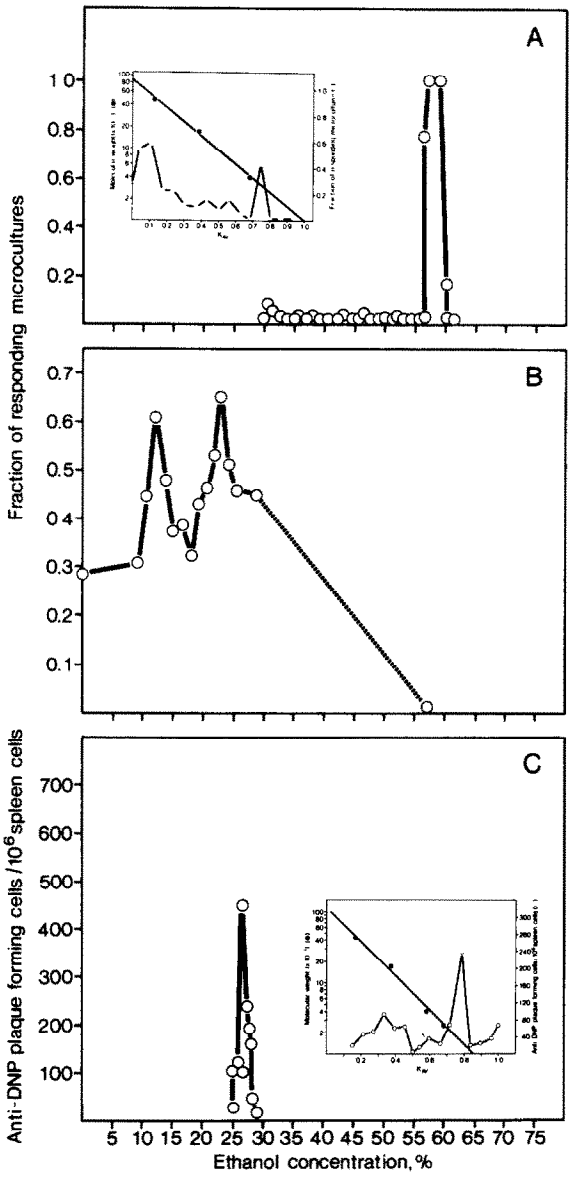

Fig.1. Reverse-phase HPLC of partially purified interleukins. The helper factors were partially purified as in section 2, injected into reverse-phase HPLC columns in 12 mM HCl and eluted with linear gradients of ethanol in the same acid. Fractions of 200–300 μl were collected and, after removal of aliquots for assays, dried in vacuum and stored in liquid nitrogen. The Con A factor was assayed by its T cell-replacing activity, and the KLH-factor by its KLH-specific helper activity. In the figure the activities are plotted as a function of the eluting ethanol concentration. (A) Con A factor pre-purified by ammonium sulfate precipitation and gel filtration (Sephacryl S-200). The insert shows the M_r distribution, in the presence of 6 M guanidine–HCl, of the biologically active factor. The procedure is described in the text. The M_r standards were: ovalbumin, 46 000; myoglobin, 17 000; β_2-endorphin, 3900. (B) Con A factor pre-purified as in (A) followed by ion-exchange chromatography (DEAE-cellulose) and gel filtration (polyacrylamide P60). $K_{av} = (V_e - V_o)/(V_t - V_o)$ where V_e = the volume of solvent required to elute the molecule of interest, V_t = the total column volume and V_o = the void volume. (C) KLH-specific factor pre-purified as the Con A factor in (B) but without the first gel filtration step (Sephacryl S-200). The insert shows the M_r distribution of the active factor, determined as in the text. The smallest M_r standard was insulin A chain at 2500.

ently the Con A factor taken through the short route of purification was considerably more hydrophobic (A) than the one pre-purified more extensively (B). Ethanol at 55–60% was required to release the factor activity from the column in panel A, but only 10–25% ethanol eluted several peaks of activity in (B). Only the assay of T cell-replacing activity is shown in the figure, but the thymocyte growth-stimulating activity pattern was identical to this.

In fig.1(C) is shown the elution profile of the KLH-specific factor. This material had been pre-treated in the same way as the Con A factor in (B) but without the gel filtration on Sephacryl S-200. The activity eluted in a narrow, but apparently not homogeneous peak at 25–30% ethanol, significantly later than the Con A factor in (B). In this connection it is of interest to mention that during ion-exchange chromatography of the two factors shown in (B) and (C), the KLH-specific factor was eluted from a DEAE-cellulose column at a lower ionic strength than that which eluted the Con A factor (not shown). This observation, considered together with the elution patterns shown in fig.1(B) and 1(C) could suggest that the KLH-specific factor is more hydrophobic than is the early-eluting (fig.1(B)) Con A factor.

The heterogeneity of the Con A factor and the KLH-specific factor was further analyzed by determining the app. M_r values of the two factors under native as well as under denaturing conditions in 6 M guanidine–HCl. Under native conditions the average app. M_r of the factor material in all 3 panels of fig.1 was ~30 000–35 000 (determined by gel filtration on calibrated columns of Sephacryl S-200 and polyacrylamide P60). A result which is in good agreement with values obtained in [1]. However, quite different results were obtained under denaturing conditions. Aliquots of the material shown in (A) and (C) were incubated for 30 min in 0.05 M Tris–HCl (pH 7.3); 6 M guanidine–HCl at room temperature and then injected, together with M_r standards, into a gel permeation column (I-125 protein column, Waters Associates). Fractionation was done by HPLC using the same buffer as above. Pools were prepared of fractions representing different M_r regions, and these were reinjected into the reverse phase RP18 column to remove the guanidine. Both factors were eluted with linear gradients of ethanol, 60–90% for the Con A factor, 25–65% for the KLH factor, and their respective activities were assayed. The inserts in fig.1(A) and 1(C) show the size distributions of the biologically active factors. Although they both had approximately the same M_r under native conditions, it is apparent that 6 M guanidine was able to separate several differently sized factor components from each other. In both cases, active material having a M_r of only 1500–3000 was seen. The highest M_r observed for active Con A factor was ~60 000 (A), but only ~20 000–30 000 for the KLH factor (C). The M_r was not determined for the Con A factor material (fig.1(B)) taken through the same steps of purification as the KLH factor, but based on their similar biochemical behavior and elution positions after HPLC, it is expected that also that factor activity will contain a mixture of high and low M_r material. The KLH factor purified through the HPLC step had retained its antigen specificity. Fig.2 shows that a significant antibody response was obtained only when DNP was bound to the appropriate carrier, KLH. It is presently not clear how such a small component can retain antigen specificity. It is the hope that further biological and biochemical studies on purified KLH factor will clarify this problem.

4. Discussion

It appears that the app. M_r values are not reliable characteristics of the mouse interleukins. Biologically active, extensively purified factors with native M_r of ~30 000–35 000 could under denaturing conditions

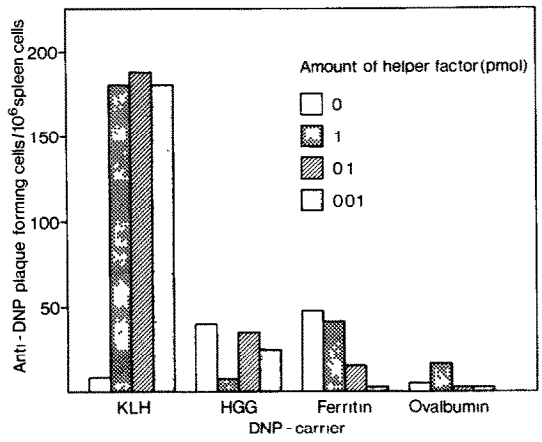

Fig.2. Carrier specificity of KLH factor. The specificity of the KLH-induced helper factor was evaluated in an in vitro assay in which the anti-DNP antibody response was measured when DNP was bound to the different carriers listed.

in acid and in 6M guanidine have M_r of 2000–60 000. Therefore, it should be expected that one factor will be able to appear in different M_r forms, depending on its environment. In fact, helper factors of different sizes have been reported [10,11], and it was suggested [12] that their app. M_r values were dependent on the factor concentration.

The heterogeneity of the two interleukins studied was most likely caused by a combination of covalent and noncovalent interactions between components of small M_r. The nature of the covalent bonds is not known, but since preliminary evidence suggests the presence of cysteine in the Con A factor, small M_r material could possibly be covalently 'aggregated' by formation of disulfide bridges. Further studies in which this possibility will be investigated are currently in progress.

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