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Multiple serum inhibitors of lectin-induced lymphocyte proliferation in nephrotic syndrome

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SUMMARY

Inhibitory activity on PHA- and Con A-induced lymphocyte proliferation was observed in the serum of 29 patients with nephrotic syndrome (NS); this inhibitory activity was present both in steroid-sensitive nephrotic syndrome (SSNS; 18 patients) and in NS due to other glomerulopathies (11 patients). In order to characterize the inhibitory activity, peripheral blood lymphocytes from normal donors were stimulated with various concentrations of Con A in culture medium supplemented with: (1) 20% SSNS serum, (2) various concentrations (1, 5 and 20%) of either SSNS serum or normal human serum (NHS) and (3) 20% of a serum prepared by mixing different proportions of SSNS and NHS. The results suggest that the inhibitory activity is due to at least two different factors: (a) inhibitor(s) acting competitively with the lectin Con A, and (b) inhibitor(s) neutralized by factor(s) present in NHS. A disturbance in the normal equilibrium between inhibiting and enhancing factors which results in overall inhibition might well be a consequence of the marked alteration in serum proteins characteristic of NS.

INTRODUCTION

Moorthy, Zimmerman & Burkholder (1976) reported that phytohaemagglutinin (PHA) induced lymphocyte proliferation was inhibited by plasma from patients with minimal-change nephrotic syndrome but unaffected by plasma from patients with nephrotic syndrome (NS) due to other glomerulopathies (GP). In fact, although the pathogenetic mechanisms of minimal-change nephrotic syndrome are still obscure, some clinical observations suggest involvement of T cell immunity. The most significant evidence is the induction of remission by corticosteroids (steroid-sensitive nephrotic syndrome) (SSNS) as well as other immunosuppressive agents such as cyclophosphamide (Barratt & Soothill, 1970). Remission may also be induced by measles (Janeway *et al.*, 1948), an infection known to depress T cell immunity (Smithwick & Berkovich, 1966). Furthermore, minimal-change nephrotic syndrome has been described in association with Hodgkin's lymphoma and in these cases evolution of the nephropathy appears related to activity of the lymphoma (Plager & Stutzman, 1971). Mainly on the basis of these observations, Shalhoub (1974) hypothesized that minimal-change nephrotic syndrome might be the result of a primary T cell disturbance. The previously mentioned finding of Moorthy *et al.* (1976) also confirmed by Sasdelli *et al.* (1980) seems to support Shalhoub's hypothesis; however, Iitaka & West (1979), Beale *et al.* (1980) and Martini & Vitiello (1980) found a serum-inhibitory effect in patients not only with minimal-change nephrotic syndrome but also with other forms of NS.

We confirm here that PHA and concanavalin A (Con A) induced lymphocyte proliferation *in*

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vitro is inhibited by serum not only from SSNS but also from NS due to other GP. Furthermore, we demonstrate that the inhibition is mediated by at least two factors: an inhibitory factor neutralized by normal human serum (NHS) and a competitive inhibitor of the lectin Con A.

MATERIALS AND METHODS

Subjects. Twenty-nine patients with NS were included in the study; 18 with SSNS and 11 with NS due to other glomerulopathies (four extramembranous GP, three membranoproliferative GP, one Schönlein–Henoch GP, one Berger's disease, one mesangial sclerosis with pseudohermaphroditism, one congenital NS). The patients ranged in age from 8 months to 20 years. All were studied in the active phase of the disease before any treatment; four with SSNS were studied several times at the beginning of a relapse during observation for eventual spontaneous remission. All patients presented marked proteinuria (> 3 g/24 hr) and had a creatinine clearance higher than 50 ml min^{-1} 1·73 m^{-2}.

Control subjects were 15 healthy adult volunteers taking no medications at the time of the study.

Blood obtained by venipuncture was divided into two samples: one was heparinized and used immediately for lymphocyte studies; the rest was allowed to clot and after sterilization by filtration on 0·22-μm Millipore filters, serum was stored at $-70°C$ until use in lymphocyte cultures. A pool of 25 normal human sera from healthy blood donors was used as a reference (pool serum) throughout the study.

Lymphocyte cultures. Lymphomononuclear cells were isolated from heparinized blood by density centrifugation on Ficoll-Hypaque and cultures were set up as previously described (Burgio *et al.*, 1975). Briefly, lymphocytes were cultured in round-bottomed microtitre plates at a concentration of $0{\cdot}5 \times 10^6$/ml in 0·2 ml of medium RPMI 1640 supplemented with glutamine (4 mM), penicillin (100 iu/ml), streptomycin (100 μg/ml) and 20%, 5% or 1% nephrotic syndrome serum or normal human serum. Cultures were set up in triplicate and stimulated with either PHA (GIBCO, M form) or different concentrations of Con A (Pharmacia Fine Chemicals, Sweden; 40, 80, 160 and 320 μg/ml); parallel cultures without mitogens served as controls.

Incubation was performed at 37°C in a humidified atmosphere of 5% CO_2, 7% O_2 and 88% N_2. Twenty hours before harvesting, 0·5 μCi of ^{3}H-thymidine (Amersham, sp. act. 2 Ci/mmol) were added to each culture well; cells were harvested by means of a multiple sample precipitator (Skatron, Norway) and radioactivity was determined using a liquid scintillation counter. Results are expressed as counts per minute (c.p.m.).

The proliferation-promoting activity of the test serum was expressed as a percentage of the response in normal serum according to the following formula:

$$\frac{\text{proliferative response (c.p.m.) in test serum}}{\text{proliferative response (c.p.m.) in pool serum}} \times 100.$$

The proliferation-promoting activity of sera from individual healthy subjects ranged from 75 to 115% for Con A-stimulated cultures and from 78 to 118% for PHA-stimulated cultures.

RESULTS

Mitogen-induced proliferation of patient's lymphocytes

Lymphocytes from five patients with SSNS and six patients with NS due to other GP responded normally when stimulated *in vitro* with PHA or Con A in the presence of NHS (mean c.p.m. $\times 10^{-3}$: PHA = 76·4 ± 18 s.d., Con A = 53·5 ± 14 s.d.

Inhibition of PHA- and Con A-induced lymphocyte proliferation by SSNS serum and serum from patients with NS due to other GP

When either normal peripheral blood lymphocytes (PBL) or SSNS lymphocytes were stimulated *in vitro* with Con A or PHA in culture medium supplemented with SSNS serum, lymphocyte

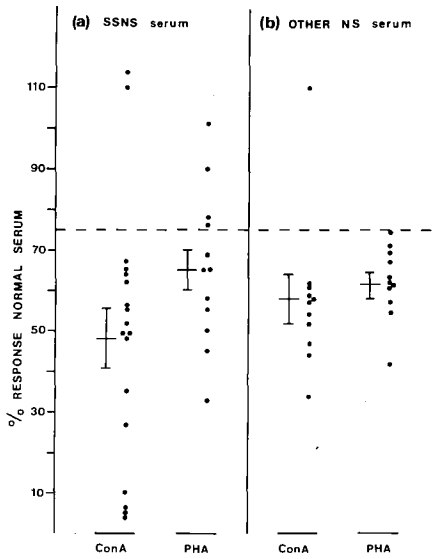

Fig. 1. Inhibition of PHA- and Con A-induced lymphocyte proliferation by steroid-sensitive nephrotic syndrome serum (a) and serum from patients with nephrotic syndrome due to other GP (b). The proliferation-promoting activity of the individual sera are reported on the ordinate as a percentage of the response in normal serum (see formula in Materials and Methods section). Mean values (*horizontal bars*) ± 1 standard error (*vertical bars*) are reported. The dashed line is the lower limit of the normal range.

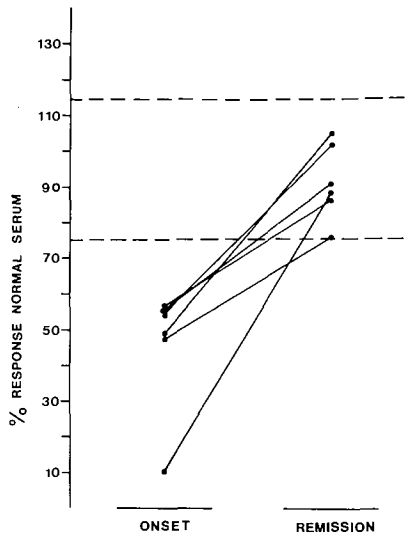

Fig. 2. Inhibition of Con A-induced lymphocyte proliferation by serum for six patients with steroid-sensitive nephrotic syndrome studied at the onset and 2–3 months after induction of remission. Dashed lines are the limits of the normal range. The proliferation-promoting activity of the individual sera are reported on the ordinate as a percentage of the response in normal serum (see formula in Materials and Methods section).

proliferation was significantly inhibited by SSNS as compared to pooled normal serum. A comparable inhibitory effect was noted when serum from patients with NS due to other GP was substituted for SSNS serum in the culture medium (Fig. 1). In both groups of patients the inhibitory effect was more pronounced when lymphocytes were stimulated with Con A than with PHA. A lymphocytotoxic effect of nephrotic serum was ruled out by the trypan blue exclusion test (data not shown). Complete disappearance of the serum inhibitory activity of both Con A- and PHA-induced lymphocyte proliferation was observed in six patients with SSNS studied 2 to 3 months after induction of remission (Fig. 2). Repeated assessment in four patients with active SSNS (presence of heavy proteinuria) showed that inhibitory activity appeared only with development of hypoproteinaemia. In fact, all inhibitory sera except one were hypoproteinaemic; however, there was no significant correlation between the degree of inhibitory effect and the concentration of serum albumin or alpha-2-globulins.

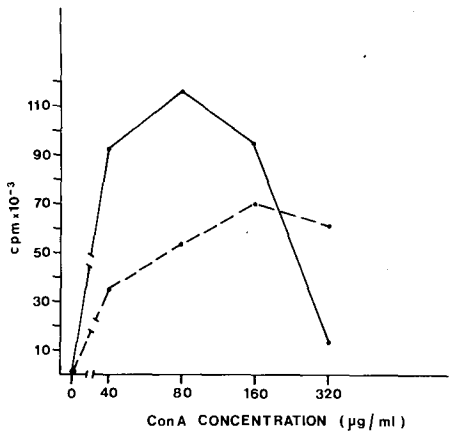

Fig. 3. Proliferative response of normal peripheral blood lymphocytes (c.p.m. $\times 10^{-3}$, ordinate) cultured either in normal human serum (NHS, *continuous line*) or in serum from patients with steroid-sensitive nephrotic syndrome (SSNS, *dashed line*), stimulated with various concentrations of Con A (abscissa). Figure shows a representative experiment.

Proliferation of normal PBL stimulated with various concentrations of Con A in the presence of SSNS serum

Normal PBL were stimulated with various concentrations of Con A in the presence of either SSNS serum (from six patients) or NHS (see Materials and Methods section).

As shown in Fig. 3, maximum proliferation was not only decreased in the presence of SSNS serum but also required a higher concentration of Con A to be elicited. This shift to the right of the dose–response curve was noted in four of the six sera tested; the other two sera caused a marked inhibition of lymphocyte proliferation (>90%) independently of the Con A concentration.

Normal PBL were stimulated with various concentrations of Con A in cultures supplemented with different concentrations (20%, 5%, 1%) of either SSNS serum (from six patients) or NHS. The lower concentration (1%) of NHS produced a shift of the dose–response curve to the left with only a slight effect on the maximum proliferative response (Fig. 4a), while with SSNS serum the maximum proliferative response was significantly higher with concentrations of 1% than with 20% (Fig. 4b).

Proliferation of normal PBL stimulated with various concentrations of Con A in the presence of mixtures of SSNS serum and NHS

Normal PBL were stimulated with various concentrations of Con A in cultures supplemented with 20% of a serum prepared by mixing different proportions of SSNS serum (five patients) and NHS. In all cases the presence of 1:4 NHS was sufficient to increase the maximum proliferative response considerably which even returned to normal in one case. Maximum proliferative response always

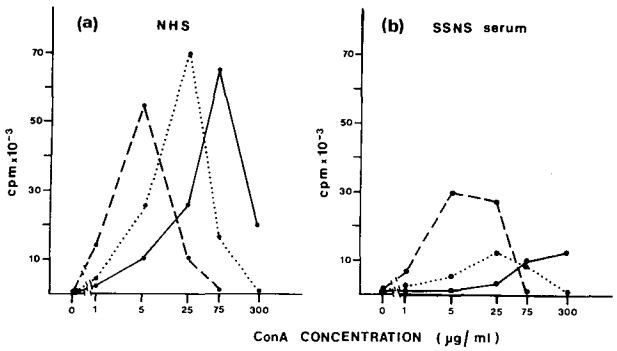

Fig. 4. Proliferative response of normal peripheral blood lymphocytes (c.p.m. $\times 10^{-3}$, ordinate) cultured either (a) in normal human serum (NHS) or (b) in serum from patients with steroid-sensitive nephrotic syndrome (SSNS), stimulated with various concentrations of Con A (abscissa). Cultures were supplemented with 1% (*dashed line*), 5% (*dotted line*) or 20% (*continuous line*) serum. Figure shows a representative experiment.

normalized when 1:2 NHS was present in the serum mixture, but always required a high concentration of Con A to be elicited; this shift of the dose-response curve became evident in those cases in which it has not been observed when cultures were supplemented with SSNS alone (Fig. 5).

DISCUSSION

The results presented indicate that lymphocyte proliferation *in vitro* is inhibited by serum from patients with SSNS as well as from patients with NS due to other GP; the inhibitory effect has been shown to result from at least two different factors: (a) inhibitor(s) acting competitively with the lectin Con A, and (b) inhibitor(s) neutralized by factor(s) present in NHS. The effect of both inhibitors appears to be reversible since lymphocyte proliferation of patients with NS cultured in NHS was normal.

The finding that the inhibitory effect of SSNS serum is not specific but is shared by serum from patients with other NS does not confirm the previous data of Moorthy *et al.* (1976) and Sasdelli *et al.* (1980) but is in agreement with the results of Iitaka & West (1979) and Beale *et al.* (1980). These

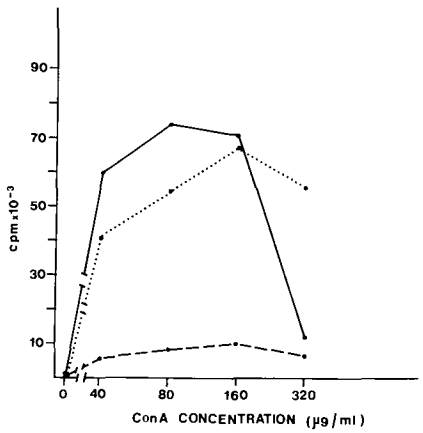

Fig. 5. Proliferative response of normal peripheral blood lymphocytes (c.p.m. $\times 10^{-3}$, ordinate), stimulated with various concentrations of Con A (abscissa) in culture medium supplemented with normal human serum (NHS, *continuous line*), serum from patients with steroid-sensitive nephrotic syndrome (SSNS, *dashed line*) or a 1:1 mixture of NHS and SSNS (*dotted line*). Figure shows a representative experiment.

discrepancies might result at least partially from differences in the culture conditions used in the various studies, as well as in the selection of patients.

Longitudinal study of some patients showed that the inhibitory activity, absent in the early phase of relapse characterized by heavy proteinuria in the absence of significant alterations in serum proteins, appeared only when the hypoproteinaemia became evident several days later. In fact, all but one sera with inhibitory activity were from patients with hypoproteinaemia; however, the degree of inhibitory activity of the individual sera was not significantly related to the levels of serum albumin or alpha-2-globulins.

These data suggest that the inhibitory effect of SSNS serum is not related to the pathogenesis of the disease but rather that it is secondary and related to metabolic abnormalities (hypoproteinaemia, hyperlipidaemia, etc.). Many substances present in normal serum are known to affect lymphocyte proliferation *in vitro* acting either as inhibitors (Ford, Caspary & Shenton, 1973; Burger, Lilley & Vettor, 1974; Occhino *et al.*, 1973; Fujii & Edgington, 1980; Waddell, Taunton & Twomey, 1976; Chiasari, 1977) or as factors promoting cell growth (Phillips & Azari, 1974; Phillips, 1978); in fact, some inhibitors such as alpha-2-macroglobulin, low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) are elevated in NS serum (Newmark *et al.*, 1975) while albumin, known to be essential for lymphocyte proliferation (Polet & Spieker- Polet, 1975), is low. Serum levels of another well-known inhibitor of lymphocyte blastogenesis, alpha-fetoprotein (Yachnin, Soltani & Lester, 1980), were normal in our patients (data not shown). To investigate the mechanism(s) whereby lymphocyte proliferation is reduced in the presence of SSNS serum we carried out experiments using Con A as a mitogen; in fact, the decrease in responsiveness induced by SSNS serum was more pronounced and constant when lymphocytes were stimulated with Con A than with PHA (Fig. 1).

Experiments with normal lymphocytes stimulated with various concentrations of Con A in SSNS serum (Fig. 3) showed that peak proliferative responses were not only lower than in NHS but also required higher mitogen concentrations to be elicited: this finding suggests that a competitive inhibitor of Con A is present in SSNS serum. Moreover, these results underline the importance of performing dose–response studies when comparing lymphocyte responses in NHS and test sera. The presence of a competitive inhibitor of Con A does not fully account for the reduced lymphocyte proliferation observed in SSNS serum; in fact, even the peak lymphocyte response obtained with very high concentrations of Con A was consistently lower than normal (Fig. 3).

The reduction of lymphocyte proliferation observed in SSNS serum and not accounted for by the presence of the competitive inhibitor of Con A might have resulted either from a lack of serum growth factor(s) or from the presence of other inhibitory factors. To test this hypothesis normal PBL were stimulated with different concentrations of Con A in cultures supplemented with different concentrations of SSNS serum. Since the lymphocyte proliferative response to appropriate concentrations of Con A increased with decreasing concentrations of SSNS serum (down to 1%), we concluded that the reduced response in SSNS serum was unlikely to result from lack of growth factors; rather, these results suggest that SSNS serum contains a true inhibitor of lymphocyte proliferation.

Experiments carried out in cultures supplemented with serum prepared by mixing SSNS serum and NHS in various ratios showed that the presence of as little as 1/4 of NHS considerably increased (and in one experiment normalized) the maximum proliferative response. This experiment can be taken as evidence for the presence in NHS of a factor neutralizing the inhibitory activity of SSNS serum. These data are in agreement with those of Iitaka & West (1979) who in idiopathic nephrotic syndrome described a serum inhibitor which has a receptor on lymphocytes and which can be blocked by a factor present in NHS.

In cultures supplemented with 1:1 mixtures of NHS and SSNS serum, the peak proliferative response was normal but required a higher than normal concentration of Con A to be elicited. From this experiment we concluded that the competitive inhibitor of Con A and the inhibitor neutralized by a factor present in NHS represent different inhibitory activities so that the overall inhibitory activity of SSNS serum results from the interaction of various mechanisms.

Beale *et al* (1980) have recently reported that serum from patients with SSNS contains a factor inhibiting the response of mixed lymphocyte cultures and apparently absent in sera from patients

with other forms of NS. Further studies would be interesting to determine whether the Con A-independent inhibitory activity identified in SSNS sera in the present study may play a role in the above-mentioned inhibition of MLC.

As previously mentioned, human serum contains inhibitors as well as growth-promoting factors of lymphocyte blastogenesis; because NS is characterized by a marked imbalance of serum proteins, it is attractive to speculate that the inhibitory effect of NS serum reported in the present study is the overall result of an altered equilibrium between inhibiting and enhancing factors which are balanced in physiological conditions.

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