

Autolymphocytotoxins in syphilis

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SUMMARY Nineteen of 23 serum samples from patients with secondary syphilis, six of 24 from patients with primary syphilis, and four of seven from patients with early latent syphilis had complement-dependent autolymphocytotoxic reactivity at 15°C. After treatment, the reactivity was decreased. It was not directed only against autologous lymphocytes but also against allogeneic lymphocytes. Sephadex G-200 gel filtration showed that the lymphocytotoxins were present in the IgM-containing exclusion peak. Furthermore, serum samples that had high antibody titres in the Reiter protein complement-fixation test or Wassermann-Kolmer reaction also had a high frequency of cold-reacting autolymphocytotoxins. This indicates that the autolymphocytotoxic reactivity is related to the humoral response in syphilis.

Introduction

In patients with early syphilis there is an extensive response by the antibody-synthesising immune system. It is known that a hyperimmunoglobulinaemia may develop,^{1,2} and antibodies of several specificities are produced. However the humoral immune response does not appear to protect the human host against progression of the disease. This has led to the investigation of the role of cellular mechanisms in the immune response to *Treponema pallidum*.

The results of such investigations may point to a disturbance of T-lymphocyte functions in patients with early syphilis. It has been found (a) that patients with early syphilis usually do not show a delayed hypersensitivity reaction to *T pallidum* antigens;³⁻⁵ (b) that a depletion of lymphocytes in the thymus-dependent areas of lymph nodes from patients with early syphilis may occur;⁶ and (c) that lymphocytes from patients with primary or secondary syphilis cultured in the presence of autologous plasma show a reduced response to phytohaemagglutinin (PHA).⁷

The investigations of Levene *et al*⁷ and Kantor⁸ have shown that plasma from patients with secondary syphilis also causes a reduction in the PHA-response of normal lymphocytes. It is suggested that plasma from patients with secondary syphilis contains an inhibiting factor which may be a treponemal product or an autoantibody directed against lymphocytes. A defect in the T-cell

population might be related to the presence of this inhibiting factor.⁷ Naito *et al*⁹ have found that sera from patients with syphilis exert a cytotoxic effect on normal lymphocytes. We have found that sera from patients with early syphilis contain a lymphocytotoxin directed against the patient's own lymphocytes and have investigated some of its properties.

Patients and methods

STUDY POPULATION

Fifty-four patients with untreated early syphilis (of less than two years' duration) were studied. The diagnosis was confirmed by darkfield demonstration of *T pallidum* or by a positive result to the *T pallidum* immobilisation (TPI) test or by both. Twenty-four of the patients had primary, 23 secondary, and seven early latent syphilis. The mean age of the patients was 29 years, with a range of 15-54 years. The sex ratio (male-to-female) was 3.5:1.

Treatment consisted of three intramuscular injections of procaine benzylpenicillin 600 000 units in oil suspension plus 2% aluminium monostearate (Almopen) a week for six weeks.

The TPI and Reiter protein complement-fixation (RPCF) tests and the Kolmer modification of the Wassermann reaction were carried out at the National Institute of Public Health, Bilthoven, the Netherlands. Serum and blood samples for the preparation of cell suspensions were obtained from 42 patients just before treatment. Serum samples were obtained from the other 12 patients before and at the end of treatment. In the latter cases, the initial and final samples were tested simultaneously by the

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microlymphocytotoxic test against lymphocytes isolated from blood specimens obtained at the end of the treatment period. The pretreatment serum samples were stored at -70°C .

CONTROLS

Serum samples and cell suspensions obtained from 18 healthy subjects were used as controls. The mean age of the controls was 29 years with a range of 18-45 years. The sex ratio (male-to-female) was 2.6:1.

PREPARATIONS OF LYMPHOCYTES

Mononuclear cells were isolated by the method of Bøyum¹⁰ with minor modifications. Peripheral blood anticoagulated with 20 IU preservative-free heparin per ml (Thromboliquine, Organon, Oss, the Netherlands) was diluted two-fold with Dulbecco's phosphate-buffered saline without Ca^{++} and Mg^{++} , pH 7.3 (PBS). Three millilitres of the diluted blood was layered on to 1.5 ml Ficoll-Isopaque (Lymphoprep, Nyegaard & Co, Oslo, Norway) in glass tubes with an inner diameter of 9 mm. After centrifugation at $800 \times g$ for 15 minutes at room temperature, the cells at the interface were harvested and washed three times with PBS-BSA (PBS supplemented with 1% w/v demineralised bovine serum albumin; Organon, Oss, the Netherlands). After the final wash, the cells were resuspended in MEM (Eagle's minimum essential medium spinner-modified; Difco Laboratories, Detroit, Michigan, USA) at a concentration of 1×10^6 cells/ml.

DETECTION OF AUTOLYMPHOCYTOTOXIC REACTIVITY

The autolymphocytotoxic reactivity of the sera was determined by the microlymphocytotoxic test described by Mittal *et al.*¹¹ The incubation time and temperature were modified according to Naito *et al.*⁹. Briefly, 1 μl of the lymphocyte suspension was incubated at 15°C with the test serum under oil in disposable microtest plates (Terasaki plates, Falcon Plastics). After 30 minutes, 5 μl of rabbit complement were added and the incubation was continued for another three hours at 15°C . After incubation, 3 μl of eosin Y dye and 8 μl of 40% formalin were added sequentially to each well. With an inverted phase-contrast microscope, the degree of cytotoxicity was assessed by estimating the proportion of stained cells and scored as follows: -, 0-4% stained; \pm , 5-14% stained; +, 15-24% stained; ++, 25-39% stained; +++, 40-79% stained; and +++, 80-100% stained.

Investigation of the sera was carried out with the following quantities and dilutions: 1 μl and 3 μl of the undiluted serum and 1 μl of the serum diluted 1/2, 1/4, 1/8, and 1/16 in MEM.

Undiluted unabsorbed rabbit serum was used as the complement source; this was selected for good complement activity and minimal cytotoxic activity under the conditions of the test. To determine whether complement was required for the cytotoxic reaction and to exclude non-specific cytotoxic effects, each serum sample (1 μl undiluted and 1 μl diluted 1/2) was also tested with heat-inactivated complement. In each test, a positive and a negative control sample was included.

SERUM FRACTIONATION

Cytotoxic sera were fractionated by applying 5 ml of serum to a Sephadex G-200 column (2.6×80 cm). The proteins were eluted with 0.1 mol/l Tris-HCl, 0.5 mol/l NaCl buffer, pH 8.6, at a flow rate of 15 ml/hr. The protein content of the eluates was monitored by measuring the absorbance at 280 nm. Three peaks were obtained. The fractions of the first (19S) peak and the second (7S) peak were pooled and concentrated to the initial volume of the serum sample by ultrafiltration using an Amicon PM-10 membrane. The immunoglobulin composition of the peaks was checked by Ouchterlony double-diffusion using anti-IgM and anti-IgG (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam). In the first peak, which was positive for IgM, no IgG could be detected whereas in the second peak, which was positive for IgG, no IgM could be detected.

The χ^2 test with Yates's correction was used to determine whether a significant association existed between high RPCF ($>1/32$) or high Wassermann-Kolmer ($>1/64$) antibody titres and a positive autolymphocytotoxic reaction.

Results

COLD-REACTING AUTOLYMPHOCYTOTOXINS

Frequency

Pretreatment serum samples from 54 patients with early syphilis and samples from 18 controls were tested for the presence of cytotoxic reactivity against autologous lymphocytes in the microlymphocytotoxic test. Some examples of the reactivity of syphilitic sera at different serum dilutions are given in table I. In most of the reactive samples, maximal autolymphocytotoxic reactivity was found with 1 μl of undiluted serum. It rarely exceeded a titre of 1/4. When 3 μl of undiluted serum was used, a prozone effect was present with all of the reactive sera tested.

The frequency distribution of the cold autolymphocytotoxic reactivity of the sera in relation to the different stages of the disease is given in table II. Each serum sample is denoted by its maximum score

TABLE I Example of the cold-complement-dependent autolymphocytotoxic reactivity of pretreatment sera from syphilitic patients at different serum dilutions

Patient No	Stage of syphilis	Serum amount and dilution					
		3 μ l 1/1	1 μ l 1/1	1 μ l 1/2	1 μ l 1/4	1 μ l 1/8	1 μ l 1/16
1	secondary	±	+	±	±	-	-
2	secondary	±	++	+	±	±	-
3	secondary	-	+	++	±	±	-
4	early latent	±	+++	++	±	-	-
5	secondary	±	+++	+++	+++	+	±

-, 0-4% stained cells; ± 5-14% stained cells; +, 15-24% stained cells; ++, 25-39% stained cells; + + +, 40-79% stained cells; + + + +, 80-100% stained cells

TABLE II Frequency of cold-complement-dependent autolymphocytotoxic reactivity in pretreatment sera from patients with early syphilis

Stage of disease	No of patients tested	Cytotoxic score*					
		-	±	+	++	+++	++++
Primary syphilis	24	12	6 (1/1, 1/1, 1/1, 1/1, 1/2, 1/2)	0	4 (1/1, 1/1, 1/1, 1/1)	1 (1/1)	1 (1/2)
Secondary syphilis	23	0	4 (1/1, 1/1, 1/2, 1/8)	4 (1/1, 1/1, 1/1, 1/2)	8 (1/1, 1/1, 1/1, 1/1, 1/1, 1/2, 1/2, 1/2)	6 (1/1, 1/1, 1/1, 1/1, 1/2, 1/4)	1 (1/1)
Early latent syphilis	7	3	0	1 (1/4)	2 (1/1, 1/2)	1 (1/1)	0
Controls	18	16	1 (1/4)	1 (1/1)	0	0	0

*Highest serum dilution showing this cytotoxic score given in brackets

-, ±, +, ++, + + +, + + + + see table I

and the highest serum dilution showing this score. The highest incidence of autolymphocytotoxins occurred in the sera of patients with secondary syphilis. Positive reactions were present in 19 (83%) of 23 of the patients. Not only were a larger number of samples reactive at this stage of the disease but the reactivity of sera from these patients against their own lymphocytes was also stronger than the autolymphocytotoxic reactivity of the sera from the patients at the other stages of the disease. Twenty-five per cent of the sera from patients with primary syphilis also showed an autolymphocytotoxic reaction. Four of seven sera from patients with early latent syphilis were cytotoxic whereas the sera from controls were inactive, except for one which showed a + reaction. When tested with heat-inactivated rabbit complement none of the reactive sera was positive.

Properties

Three of the pretreatment serum samples were tested both at 15°C and at 37°C. When the test was performed at 15°C, autolymphocytotoxic reactivity was present in two of these samples, both showing a ++ reaction. No reactivity was found when the test was carried out at 37°C.

Pretreatment serum samples from eight syphilitic patients containing cytotoxic reactivity against autologous lymphocytes were also tested against allogeneic lymphocytes. All of these samples also gave positive reactions against allogeneic cells.

The results obtained in the cytotoxic test for three pretreatment sera fractionated by elution from Sephadex G-200 are given in table III. In two cases, whole serum and the serum fractions were tested against autologous lymphocytes. Whole serum and fractions of the third patient were tested against allogeneic lymphocytes obtained from a healthy donor, since the patient was no longer available for study. The gel filtration experiments showed that the lymphocytotoxic reactivity was located in the IgM-containing exclusion-peak.

Heating the sera and the IgM-containing peak obtained by gel filtration for 30 minutes at 56°C caused a decrease in cytotoxic reactivity.

EFFECT OF TREATMENT

Serum samples obtained from patients at the end of treatment and before treatment were tested simultaneously against autologous lymphocytes isolated from peripheral blood obtained at the end of treatment. The post-treatment samples had a lower

TABLE III *Lymphocytotoxic reactivity of whole sera and Sephadex G-200 fractions*

Patient No	Serum or eluate	Serum amount and dilution					
		3 μ l 1/1	1 μ l 1/1	1 μ l 1/2	1 μ l 1/4	1 μ l 1/8	1 μ l 1/16
1*	Serum	++	++++	++++	+++	+	±
	19S peak	++	++	±	-	-	-
	7S peak	-	-	-	-	-	-
2*	Serum	±	++	+++	++	+	±
	19S peak	±	+	+	+	±	±
	7S peak	-	-	-	-	-	-
3†	Serum	+	+++	++	+	±	-
	19S peak	+	+	+	±	-	-
	7S peak	-	-	-	-	-	-

*Whole serum and fractions were tested simultaneously against autologous lymphocytes

†Whole serum and fractions were tested simultaneously against allogeneic lymphocytes obtained from a healthy donor

-, ±, +, ++, +++, +++++ see table I

cytotoxic score than the pretreatment samples (table IV).

ASSOCIATION WITH RPCFT AND KOLMER REACTION

Most sera with titres above 1/32 in the RPCFT showed a positive reaction in the cytotoxic test (20 out of 24) whereas most sera with RPCF titres \leq 1/32 gave negative results in the cytotoxic test (21 out of 30). This association was statistically significant ($\chi^2 = 13.18$; $P < 0.001$). A similar association was present between the cytotoxic reaction and the titres in the Kolmer test. When looking at sera with titres $>$ 1/64, 22 of 31 showed a positive cytotoxic reaction. Sera with titres \leq 1/64 showed a negative reaction in 16 of 23. Here also a statistically significant association ($\chi^2 = 7.17$; $P < 0.01$) was found.

Discussion

Cold complement-dependent lymphocytotoxins (CLC) reacting optimally at 15°C are found in a great number of infectious diseases,¹²⁻¹⁴ autoimmune

diseases,^{9, 15-17} after vaccination,¹⁸ in pregnancy, and in syphilis.⁹ Although in most investigations panels of allogeneic lymphocytes are used as target cells, the assumption has been made that CLC are also autolytotoxic.

This assumption has also been made for syphilis by Naito *et al.*⁹ In this study, the autolytotoxic nature of the syphilitic lymphocytotoxins was formally demonstrated. Moreover, we found that these lymphocytotoxins were more frequently present in the secondary than in the primary stage of the disease.

A characteristic common to the lymphocytotoxins found in several diseases is that they are present only at a very low concentration and that there is an optimal serum dilution at which they can be detected.¹⁹ This also applies to the syphilitic autolytotoxic lymphocytotoxins. For most of the reactive sera the autolytotoxic reactivity could be detected or showed its maximum score at 1 μ l of undiluted serum. When 3 μ l of undiluted serum was used, we found a prozone effect. This result may be explained by assuming that the occupation-density of the

TABLE IV *Effect of treatment on the autolytotoxic reactivity in sera from patients with early syphilis*

Patient No	Stage of syphilis	Pretreatment serum		Post-treatment serum	
		Cytotoxic score	Serum dilution*	Cytotoxic score	Serum dilution
1	Primary	±	1/2	-	-
2	Primary	-	-	-	-
3	Primary	-	-	-	-
4	Primary	+++	1/1	±	1/1
5	Primary	++	1/1	±	1/1
6	Primary	-	-	-	-
7	Primary	++	1/1	±	1/1
8	Primary	++++	1/2	+++	1/2
9	Secondary	±	1/1	-	1/1
10	Secondary	+	1/1	±	1/1
11	Secondary	++	1/1	+	1/1
12	Early latent	-	-	-	-

*Highest serum dilution showing this cytotoxic score

-, ±, +, ++, +++, +++++ see table I

lymphocytotoxins on the lymphocyte surface membrane displays an optimum where good complement activation is achieved.

In view of the complement dependence of the syphilitic autolymphocytotoxin(s), it seems likely that it is an antibody and its presence in the 19S exclusion peak after Sephadex G-200 chromatography suggests that it is IgM.

The question whether or not the syphilitic lymphocytotoxins are specifically directed against certain subpopulation(s) of lymphocytes cannot be answered yet. However, in view of the fact that sera from 10 of our syphilitic patients killed 40% to almost 100% of the cells in the microlymphocytotoxic test, it is likely that the thymus-dependent lymphocytes are also involved as target cells.

Since the syphilitic lymphocytotoxins can only be detected at low temperatures, their biological function is not clear. However the possibility that they do react with lymphocytes at 37°C at a very low concentration (which may be too low to cause a cytotoxic reaction but at which they are able to influence the immune reactivity of the lymphocytes *in vivo*) cannot be excluded.

The depletion of lymphocytes in the thymus-dependent areas of lymph nodes that has been found in 11 of 18 patients with early syphilis⁶ may be the result of the reactivity of lymphocytotoxins, working by a mechanism different from that operating at 15°C in the microlymphocytotoxic test. It may be that in spite of the temperature of 37°C the lymphocytotoxins react cytotoxically in the lymph nodes, helped by the influence of environmental factors. Another possibility is that a minimal non-cytotoxic coating of lymphocytes with lymphocytotoxins exists at 37°C, which may enhance phagocytosis of the lymphocytes. The last mechanism fits the results of Turner and Wright,⁶ who found a replacement of lymphocytes by macrophages in the paracortical areas of lymph nodes.

Possibly, a relation exists between the presence of lymphocytotoxins in syphilitic sera and the inhibition of the PHA-response caused by plasma from these patients.⁷ This inhibition was found with autologous as well as with allogeneic lymphocytes and was greater with plasma from patients with secondary syphilis than with plasma from patients with primary syphilis. This parallels our finding that the lymphocytotoxins react with autologous as well as with allogeneic cells and that they are more frequently found and show higher cytotoxic scores in sera from patients with secondary syphilis than in sera from patients with primary syphilis.

It appeared that sera with high antibody titres in the RPCFT or Kolmer reaction also had a high

frequency of cold autolymphocytotoxins. This indicates that the autolymphocytotoxic reactivity is related to the humoral immune response to *T pallidum*. If the autolymphocytotoxins regulate the immune response by the suppression of the immune reactivity of the thymus-dependent lymphocytes, the relation between the autolymphocytotoxic reactivity and the humoral immune response might explain the inverse relation between the humoral and the cell-mediated immune response in primary and secondary syphilis.

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