Cell-mediated immune response in gonorrhoea

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There is a high incidence of gonorrhoea in many parts of the world. As time passes, the disease becomes more of a problem partly because of our inability to contain it by social methods (Holmes, Counts, and Beaty, 1971) and also because complications appear to be arising with increasing frequency. An attack of gonorrhoea confers no detectable protection to the host against subsequent exposure to the disease. Many workers have therefore been prompted to investigate the host response to the causative organism, *Neisseria gonorrhoeae*.

This bacterium, which is an intracellular parasite, induces in the infected patient an antibody response of circulating immunoglobulin G, and to a lesser extent immunoglobulin M (Cohen, Kellogg, and Norins, 1969), and also of local secretory immunoglobulin A. The presence of delayed hypersensitivity in gonorrhoea was first demonstrated by skin-testing methods at the beginning of the century (Teague and Torrey, 1907; Torrey, 1907). It occurred during the second week of infection and increased in incidence and strength of reaction with the duration of infection or the presence of clinical complications. Although cell-mediated delayed-type skin hypersensitivity has long been known to occur, only recently has the technique of lymphocyte transformation been used to demonstrate the existence of a cell-mediated immune response (Kraus, Perkins, and Geller, 1970). To verify the suggestion that such a cell-mediated immune response exists in gonorrhoea, the lymphocyte transformation technique has been used in testing a small number of patients with gonorrhoea and controls without the disease, using in the test gonococcal and non-gonococcal antigens.

Material and methods

Specimens were obtained, as described later, from fourteen patients with confirmed gonorrhoea: nine cases of acute gonorrhoea in men and women treated early in

the infection; three cases of complicated infection; and two cases of repeated infection. In one further case the aetiology was doubtful.

Specimens from ten patients without gonorrhoea were tested as controls, in addition to the controls used routinely in the lymphocyte transformation test.

Several batches of gonococcal antigen were used; these were prepared by different methods from old laboratory strains of the organism. Some of the sera were also tested against *N. meningitidis* and salmonella antigens.

Lymphocyte transformation technique

The test depends upon the ability of lymphocytes from sensitized patients to 'transform' into blast cells *in vitro* after contact with the sensitizing antigen. The number of 'transformed' cells was counted indirectly by labelling with C^{14} thymidine, which is incorporated into newly formed DNA and is thus a measure of 'blast' cell formation. The results were expressed as the stimulation index, this being the ratio of the counts per minute of the antigen-stimulated transformation to the counts per minute of the unstimulated control.

20 ml. of patient's blood were collected in heparinized universal containers, and also 10 ml. of blood into a plain tube. 3 per cent. dextran was added to three parts of heparinized blood to give a final concentration of 25 per cent. 5 ml. aliquots of this mixture were put into sterile tubes and incubated at 37°C. for 1 hr.

The supernatants containing plasma and cells were transferred into further sterile tubes (three supernatants per tube) and spun at 1,200 r.p.m. for 10 min. The plasma was discarded and each pellet re-suspended in 1 ml. of Medium 199, made up to 6 ml./tube. These were then spun at 1,200 r.p.m. for 10 minutes and the supernatant again discarded.

The cells were re-suspended to a volume of 3 ml. (approx. 1 ml./tube) in Medium 199, and 0.1 ml. of the suspension was placed in 0.8 ml. of Medium 199 with 0.1 ml. Trypan blue, and counted. The remainder was diluted with Medium 199 to give 10^6 cells/ml. Serum was added to give a final concentration of 10 per cent. in the diluted cell suspension. 1 ml. aliquots of these cell suspensions were used, and 0.1 ml. of antigen per tube added. Phytohaemagglutinin (PHA) was diluted one in ten, in Medium 199, and used in 0.1 ml. aliquots in positive controls. No antigen was added to negative controls. 5 per cent. CO_2 /95 per cent. air was passed through the

tubes for a few seconds, and the lids screwed on. The tubes were then incubated for 3 days.

Late on the third day, 0.1 ml. C^{14} thymidine (0.25 μ Ci) was added to each tube and the cultures were re-incubated overnight at 37°C. They were then centrifuged at 1,000 r.p.m. for 6 min. 1 ml. of normal saline at 4°C. was added to each, and after re-suspending the cells 1 ml. of 10 per cent. trichloroacetic acid was added. The suspensions were filtered through 2-cm. diameter glass-fibre filter pads. The tubes were washed with 5 per cent. trichloroacetic acid, which was also poured through the filters, and the pads were washed with 2 $\times$ 10 ml. AR methanol. The pads were then put into counting vials and dried at 60°C. without lids. To these dried pads were added 6 ml. toluene scintillation fluid containing 5 ml. hydroxide of hyamine per litre. The disrupted pads were then counted in a Tricarb liquid scintillation counter by standard methods.

For histological examination, the cells were re-suspended in 1 ml. normal saline, but, instead of adding trichloroacetic acid, 5 ml. of aceto-methanol (one part acetic acid to 3 parts AR methanol) were added, and the cells spun at 1,000 r.p.m. for 5 min. Each pellet of cells was re-suspended in 1 ml. aceto-methanol. Two smear preparations were made from each tube, air-dried, and stained with Jenner-Giemsa stain, and finally mounted for differential cell counting. This was done in order to ensure correlation with the C^{14} thymidine counting results and to observe the state of the cells in respect of toxicity, etc. The results were as expected and the preparations satisfactory.

Preparation of antigen

The strains of *Neisseria gonorrhoeae* used for antigen production were from freeze-dried old laboratory cultures designated CN 890 and CN 1000. These were originally isolated in 1943 and 1944. No Type 1 or 2 colonies appeared when these were grown on enriched gonococcal culture medium (Kellogg, Cohen, Norins, Schroeter, and Reising, 1968). Cultures were grown on Thomson flats of agar containing acid-hydrolysed casein and yeast extract, inoculated with the growth from one slope of blood agar containing 200 μ g./ml. cystein, and incubated for 40 hrs at 37°C. The growth was washed off the agar with sterile saline to yield approximately 25 ml. of a suspension with a density equivalent to Brown's Tube 5 (*ci* 4.5×10^9 organisms/ml.). For individual antigen preparations the harvest from one Thomson flat of each strain was pooled and processed as described. Aseptic precautions were used throughout the preparation of the antigens and no preservatives were added.

Antigens A and B were prepared by a modification of the method described by Price (1933). After the bacteria had been washed twice in sterile saline, the pH was adjusted to 10.0 using 1N sodium hydroxide and the suspension was incubated at 37°C. for 2 hrs. The pH of the supernatant collected after centrifuging at 5,000 G for 45 minutes was adjusted to 5.0, using 12 per cent. w/v trichloroacetic acid, and incubated at 37°C. for 20 min. The pellet obtained after centrifugation at 5,000 G for 45 min. was re-suspended in 5 ml. sterile saline and the

pH was adjusted to 7.0 using 0.1 N sodium hydroxide. The resulting suspension was used as Antigen B, and Antigen A was a similar suspension which had been passed through a sterile 0.2 μ membrane filter. The latter procedure results in a depression of complement-fixing activity in the antigen.

For Antigen C, cells were washed and re-suspended in one half volume of phosphate buffer, pH 7.0. The suspension was sonicated at 25 kilocycles/second at 10 μ amplitude in a MSE 100W ultrasonic disintegrator for 2 mins. and unbroken cells and large debris were removed by centrifugation at 5,000 G for 20 min.

Antigen D was prepared by a buffer extraction procedure. Cells were washed in 0.05 M glycine-HCl buffer, pH 2.6, and centrifuged at 10,000 G for 20 mins. The pellet was re-suspended in 0.05 M tris-HCl buffer, pH 9.0, and the supernatant after centrifugation at 100,000 G for 2 hrs was used as Antigen D.

Antigen E was prepared from the pellet remaining after the preparation of Antigen C, using the treatment described for Antigen D.

A control antigen was made from a culture of *Neisseria meningitidis* Group D (CN 3327) by sonicating a suspension of cells as described for Antigen C. In this case the cells were obtained by suspending the growth from a Thomson flat of blood agar, after 24 hrs' incubation at 37°C. in 20 ml. sterile saline.

A sonicated suspension of *Salmonella adelaide* (CN 1430) was also used as a control antigen. The culture was grown on a Thomson flat of nutrient agar at 37°C. for 16 hrs and harvested in 20 ml. sterile saline.

Results and conclusions

The results are presented in the Table. A transformation ratio index of 3.0 or above was considered significant, and termed positive. In patients with gonorrhoea recently treated or untreated, positive transformations occurred in twelve, and were absent in two. Of the ten specimens taken from control cases, four were positive and six were negative. The transformation indices, however, were much higher in the gonococcal cases, and comparatively low in the control cases with one exception (Table).

Detailed histories showed that, of the men with acute gonorrhoea who were tested, Case 1 (negative) had his infection for only 2 days on each occasion before treatment; Case 2 (positive) for 4 to 5 days before treatment; Case 3 (positive) for an unspecified length of time; Case 4 (positive) for 2 to 3 days in some attacks, and for a week or so in others, in the several attacks during the months preceding the test.

Of the women with gonococcal cervicitis who were tested, Case 6 (negative) had been infected twice by her husband and obtained treatment within 3 days of symptoms occurring; Case 7 (positive) attended a clinic a week or so after the onset of symptoms;

TABLE *Results of transformation tests in patients with gonorrhoea and controls*

Series	Case no.	Sex/ Age	Antigen							PHA	History
			N. gonorrhoeae					N. meningitidis	Salmonella		
			A	B	C	D	E				
Gonorrhoea	1	M/27	1.3	2.1	—	—	—	—	—	38.0	Three treated acute attacks of gonorrhoea; all presumably cured: 1970, 1971, and 1972
	2	M/24	1.9	99.4	—	—	—	—	—	1.4	Acute gonorrhoea; treated and cured: 1971
	3	M/23	—	1.7	7.1	2.0	5.9	—	—	38.0	Subacute gonorrhoea; finally treated and cured: 1970
	4	M/27	—	6.1	29.4	3.1	24.2	—	—	263.0	Repeated attacks of gonorrhoea from his wife; husband treated and cured
	5	M/30	—	—	3.7	—	—	—	—	122.0	Cystitis thought to be due to gonococci-morphological; not in culture
	6	F/26	—	—	1.6	—	—	0.8	0.7	140.7	Gonococcal acute cervicitis (1971, 1972) from husband; treated and cured in early stages
	7	F/20	3.5	4.1	—	—	—	—	—	14.5	Gonorrhoea, acute; contact of friend: 1971
	8	F/40	—	—	11.6	—	—	1.3	0.8	185.0	Gonococcal acute cervicitis (1971) from husband
	9	F/43	2.9	16.2	—	—	—	—	—	83.3	Gonococcal acute cervicitis
	10	F/28	1.8	10.5	—	—	—	—	—	34.0	Gonorrhoea, acute, with little change in cervix
	11	F/24	—	1.8	39.5	29.3	37.4	0.4	8.5	35.0	Gonococcal salpingitis, subacute; treated recently
	12	F/20	—	—	0.8	1.0	—	0.9	0.7	19.0	Said by G.P. to be salpingitis: no clinical evidence; no bacteriological evidence of gonorrhoea
	13	F/25	—	4.2	2.2	2.3	10.7	0.55	0.55	20.0	Chronic salpingitis and tonsillitis
	14	M/27	—	1.3	12.0	2.0	11.0	2.2	21.9	100.0	(Re-infecting husband and wife: wife (15) seen with six infections; husband (14) with ten infections)
	15	F/28	—	1.0	4.3	9.4	26.2	0.6	13.3	73.0	
Controls	a	M/30	—	12.9	—	—	—	—	—	136.0	No special history sought. Operation for hiatus hernia: 1971
	b	M/20	—	3.9	—	—	—	—	—	275.0	Genital thrush: 1970, 1971
	c	M/59	—	—	1.1	—	—	0.25	0.3	137.0	Early syphilis: 1971
	d	M/32	—	—	2.6	—	—	0.3	0.35	177.0	Treated early syphilis
	e	M/—	—	—	0.5	—	—	0.2	0.35	89.0	Coronary insufficiency
	f	M/—	—	—	3.1	1.5	5.2	7.9	8.1	109.0	Random patient sent by G.P. to hospital for haemoglobin estimation. No history
	g	M/36	—	—	2.6	2.2	2.1	1.3	8.5	94.0	ESR = 23 mm./hr
	h	M/61	—	—	1.4	2.6	1.2	1.4	10.6	43.0	Angina
	i	M/44	—	1.0	1.7	1.6	2.3	9.1	6.7	44.0	Claudication
	j	M/28	—	3.5	—	—	—	—	—	337.0	Inguinal hernia
											Arthritis

Figures refer to 'stimulation indices'; 3.0 and above are read as positive
— = test not done

Case 8 (positive) was infected by her husband, probably eight or more days before attendance; in Cases 9 and 10 (positive) the infection was of unspecified duration.

Both case 11, of substantiated gonococcal salpingitis and Case 13, of probable post-gonococcal chronic salpingitis with gonococcal tonsillitis, gave a positive result, whereas Case 12, misdiagnosed as gonococcal salpingitis, gave a negative result.

Cases 14 and 15, a husband and wife (of whom the wife was a suspected carrier) who repeatedly infected each other were both positive.

In control tests, using the antigen from *N. meningitidis*, the results were negative in every case of gonococcal infection (Table).

Thus, using gonococcal antigen, positive stimulation was obtained in the majority of gonorrhoea patients (85 per cent.), some of whom had had their infection for only a few days. When treatment

had been given 2 to 3 days after infection, the results of the test were negative. In contrast, results were consistently positive when a patient had had gonorrhoea for only 4 to 5 days before treatment. In mice infected with *Listeria monocytogenes*, hypersensitivity may first be detected on the 4th or 5th day after infection (Mackness, 1962). Using the technique of lymphocyte transformation, a cell-mediated response can be detected in gonorrhoea after a similar interval of time.

It is of interest to note that Antigen B induces lymphocyte transformation more effectively than Antigen A. This finding correlates with the removal of complement-fixing activity by the final filtration step in the preparation of Antigen A.

It was reported by Kraus and others (1970) that some non-specific stimulation of lymphocytes occurred in many instances in this test. Although some non-specific stimulation was found in the

present study, there was a definite pattern of specific transformation in the more regular occurrence of positive transformation in the cases of gonorrhoea, particularly in the higher indices attained in gonorrhoea patients compared with controls.

The results favour the view that a cell-mediated immune response develops in gonorrhoea, although it seems to offer little or no protection for the patient (see Case 14). Cell-mediated immunity may have played an important role in limiting the attack in the days before specific therapy was available, when acute infections in many men continued for some 6 weeks to 2 months before spontaneously subsiding.

Summary

Using the lymphocyte transformation technique, a series of patients was investigated for the ability of lymphocytes to transform after contact with specific antigen. There were fourteen cases of gonorrhoea and ten patients without gonorrhoea: in twelve of the fourteen cases with gonorrhoea, a positive transformation ratio of 3.0 or above was obtained; and in four out of ten cases without gonorrhoea positive ratios were noted, but generally of a lower order than those found in the former group. A cell-mediated immune response was thus demonstrated, although it was also noted that a non-specific effect occurred in certain instances. It seems that the effectiveness of this immune response is small in practice and does not protect patients against further infection with gonorrhoea.

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Réponse immunitaire d'origine cellulaire dans la gonococcie

SOMMAIRE

Chez une série de malades on rechercha, en utilisant la technique de la transformation des lymphocytes, la capacité des lymphocytes à se transformer après contact avec l'antigène spécifique. Il y avait 14 cas de gonococcie et 10 malades sans gonococcie: pour 12 des 14 cas gonococciques, on obtient un rapport positif de transformation de 3.0 ou plus. Pour 4 des 10 cas non gonococciques, des rapports positifs furent notés mais généralement à un niveau inférieur à ceux du groupe précédent. Une réponse immunitaire d'origine cellulaire fut ainsi démontrée quoique l'on notât aussi, dans certains cas, un effet non spécifique. Il semble que l'action de cette réponse immunitaire est faible en pratique et qu'elle ne protège pas les malades contre une infection gonococcique ultérieure.