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IMMUNOLOGICAL SEX DIFFERENCES*

A STUDY OF PATIENTS WITH RHEUMATOID ARTHRITIS, THEIR RELATIVES, AND CONTROLS

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The hypothesis that rheumatoid arthritis is in part a manifestation of some peculiarity of the immunological mechanism has often been tested—but as yet inconclusively—in investigations which compared the immunological responses to antigenic stimuli in patients and controls. But the corollary of this hypothesis, that the peculiarity, if it exists, must be unequally distributed between the sexes has received very little attention. Because the incidence of rheumatoid arthritis is much greater in women than in men the sex ratio has often not been balanced in previous studies.

In the investigation reported here immunological responses following the injection of brucella vaccine were investigated but subjects were so chosen as to allow comparisons to be made not only between rheumatoid patients and controls but also between the sexes. Relatives of rheumatoid patients frequently exhibit immunological abnormalities similar to those found in patients, and therefore available first-degree relatives were included in the investigation which was evaluated on a statistical basis.

Brucella vaccine was chosen as antigen, because this organism was not likely to have been encountered previously by the majority of our subjects, and because the antibody produced in response to a primary stimulus is known to be a macroglobulin.

*A preliminary account of the findings reported here was presented to a meeting of the Heberden Society on June 17, 1967. An abstract and the discussion which followed was published in the *Annals* (1968), 27, 285.

Material and Methods

Selection of Subjects

Patients had classical or definite rheumatoid arthritis in accordance with the American Rheumatism Association criteria. The majority were attending the Royal Free Hospital, but some were drawn from the Departments of Physical Medicine of the Middlesex and North Middlesex Hospitals. All were seen by one of us and a detailed personal and family history was taken in each case. Hospital notes and *x*-ray films were reviewed and cases in whom the classification of the disease was doubtful were re-examined by two of us. Selected controls were matched to the patients for sex and for age within 5 years, except for women over 65 where matching age was within 10 years. Controls were members of staff of the Royal Free Hospital or outpatients receiving physiotherapy for non-inflammatory conditions. No person was included as a control who had chronic inflammatory disease, a history of hepatitis, or a personal or family history suggestive of rheumatoid arthritis or other connective tissue disease. The patient's first-degree relatives were free from clinically obvious disease. All participants were Caucasian. The composition of groups and the sex and age distribution are shown in Table I.

Brucella Injection

The brucella vaccine[1] contained 2,000 million killed organisms of *Brucella abortus* and *melitensis* per ml. Each participant received a single subcutaneous injection of 0·5 ml. into the upper arm.

[1]Parke, Davis, and Co., Detroit.

TABLE I
SEX AND AGE OF SUBJECTS

Group	Females			Males			Total	
	No.	Age (yrs) Mean	Range	No.	Age (yrs) Mean	Range	No.	Mean Age (yrs)
Patients	52	49·8	21-78	51	53·6	29-75	103	51·7
Relatives	57	43·1	15-77	34	36	14-73	91	39·5
Controls	52	48·6	23-68	54	52·9	22-80	106	50·7
Total	161	47·1	15-78	139	47·5	14-80	300	

No serious side-effects were observed. Sixty showed no reaction to the vaccine, 214 experienced mild local pain and arm stiffness lasting 1 to 3 days accompanied by varying degrees of redness and swelling at the injection site, and 26 had mild generalized malaise for 1 to 2 days. A few had transient arthralgia. Fourteen controls complained of generalized symptoms but only six patients and six relatives were thus affected.

Two separate batches of the same vaccine had to be used because the manufacturer's expiry date on the first batch lapsed before all subjects could be injected; 192 received batch I and 108 batch II. Statistical analysis revealed no significant differences in antigenic potency between the batches.

Collection and Storage of Specimens

Venepunctures were carried out immediately before the injection and at weekly intervals thereafter on four occasions.

Blood was collected into plain polystyrene containers, apart from 2 ml. placed in a citrate bottle for erythrocyte sedimentation rate (ESR) measurements.

Sera were separated within 24 hours and stored at −20°C. A small portion of each serum was stored separately for the immunodiffusion tests alone, so that these tests would be performed with serum that had been frozen and thawed a minimal number of times.

Investigations

(1) Brucella-agglutinating antibodies
(2) Brucella-blocking antibodies[2]
(3) Rheumatoid factors
  (*a*) Sheep cell agglutination test
  (*b*) Latex test
(4) Immunoglobulins
  (*a*) IgM
  (*b*) IgG
  (*c*) IgA
(5) Antinuclear antibodies
(6) Erythrocyte sedimentation rate
(7) Antistreptoloysin-O antibodies
(8) Thyroid antibodies
  (*a*) Antithyroglobulin antibodies
  (*b*) Antimicrosomal antibodies

In addition, the first sample of blood was investigated by serological tests[2] for venereal disease and throat and nose swabs[2] were sent for bacteriological examination.

Laboratory Methods

In each investigation all specimens from any one subject were tested simultaneously. Standard controls were included in each test and to avoid laboratory bias the order of the specimens was randomized. Individual tests were read throughout by the same observer who did not know the origin of specimens.

Brucella-Agglutinating Antibodies.—The agglutination technique described by Bradstreet, Bensted, Taylor, Carpenter, and Anderson (1961) was used, but the period of incubation at 37°C. was extended to 48 hours, as it was found that the agglutination was more easily assessed after this time. The antigen was a concentrated 0 suspension of *Brucella abortus*.[3]

Brucella-Blocking Antibodies.—Antiglobulin tests were done by the method of Wilson and Merrifield (1951) modified by Kerr, Coghlan, Payne, and Robertson (1966).

Sheep Cell Agglutination Test (SCAT).—This was performed in MRC agglutination trays, based on the technique of Ziff, Brown, Lospalluto, Badin, and McEwen (1956), but sera were absorbed once only with an equal volume of sheep cells,[4] and the basic agglutinin titre was read after 2 hours' incubation at 37°C. Cells were sensitized to 50 per cent. of the BAT. Three standard positive sera of differing titres and a negative control were included in every batch of sera tested. To detect agglutination the cells were re-suspended by gentle stirring. Agglutination at a dilution of 1/32 was regarded as the minimum positive titre.

Slide Latex Test.—This was performed using reagent[5] according to the manufacturer's instructions. Flocculation of latex by sera diluted 1/20 was considered positive.

Immunoglobulins.—As this was essentially a comparative study, no attempt was made to prepare pure sample of immunoglobulins to serve as standards for the immunodiffusion techniques, and values were related to commercially available standards (Hyland Laboratories).

Immunoglobulin M (IgM).—The modification by Claman and Merrill (1964) of the original technique of Oudin (1952) was employed. The specificity of the antiserum was checked by immunoelectrophoresis in agar. The length of the zone of precipitation was recorded at intervals over a period of several days to check the relationship between precipitation zone length and square root of the time, before deciding to read the tests after 72 hours. A series of dilutions of a serum rich in IgM was set up to check the linearity of the relationship between log concentration and length of precipitation zone. With each batch of test serum six replicates of standard sera were set up. Results were calculated by Oudin's method, obtaining the antigen equivalent of the antiserum agar by extrapolation for a series of dilutions of the standard serum. Development of precipitation zones took place at 30°C., and tests were read using an optically superimposed scale and low-power microscope.

Immunoglobulin G (IgG).—This is the only immunoglobulin which can be estimated by both chemical and immunodiffusion methods. As each of these techniques has relative advantages and disadvantages it was decided to use both.

[1]Carried out in the Department of Bacteriology of the Royal Free Hospital.
[2]Supplied by the Central Public Health Laboratory, Colindale.
[3]Sheep cells were obtained from Burroughs Wellcome and Co.
[4]From Stayne Laboratories Ltd.

The modified rivanol method estimates only a portion of the IgG, defined by the chromatographic conditions employed, but with a better standard of reproducibility than that of the single radial diffusion technique. The latter method, although detecting all of the immunologically defined IgG is influenced, to an indeterminate extent, by the varying levels of antigenically distinct allelomorphs in each sample.

(*a*) MODIFIED RIVANOL METHOD.—This was a development of the method of Saifer and Gerstenfeld (1962). After precipitation of most serum proteins with rivanol the supernatant containing IgG, transferrins, and small quantities of several other proteins, was passed through a column of CM cellulose equilibrated at pH 6·3 with a 0·0075 molar phosphate buffer. After a further wash with this buffer, only IgG was retained and was subsequently eluted with a buffer of higher pH and molarity. The eluted protein was estimated by the biuret method of Lospalluto and Ziff (1959). Salt-free chromatographically-prepared IgG was used as a standard. Reproducibility of 35 duplicate samples (95 per cent. confidence limits, coefficient of variation × "*t*") was ± 9.1 per cent.

(*b*) SINGLE RADIAL DIFFUSION.[6]—The method of Mancini, Vaerman, Carbonara, and Heremans (1964) was employed, as modified by Fahey and McKelvey (1965). Preliminary tests with serum dilutions showed that a period of diffusion of 20 to 24 hours at a temperature of 4°C. gave a straight line relationship between ring diameters and log of the antigen concentration. Duplicate sets of standards were run with every batch of tests and the antigen concentration was read from the line given by these standards. Ring diameters were read to the nearest 0·1 mm., using a mechanical stage with Vernier scale as a travelling microscope. For IgG the reproducibility of 35 duplicate sample (95 per cent. confidence limits × "*t*") was ± 28·3 per cent.).

Immunoglobulin A (*IgA*).—This was estimated by the single radial diffusion method used for IgG.

Antinuclear Antibodies (*ANF*).—The indirect immunofluorescence technique of Holborow and Johnson (1964) was used with two modifications: rat liver snap-frozen in isopentane at −70°C. was used as substrate, and a 3-minute period of fixation in absolute ethanol at room temperature was inserted before placing fluorescein labelled antiserum on the tissue. The same batch of antiserum was used throughout. It was raised in rabbits given repeated intravenous injections of a globulin fraction of human serum prepared by half saturation with ammonium sulphate, and conjugated with fluorescein by the method of Marshall, Eveland, and Smith (1958). Slides were viewed with a Reichert Zetopan microscope, employing Zeiss exciter filters UG5/3 and BG38 and a Wratten 2B barrier filter. Sera were tested at dilutions of 1/10, 1/40, 1/160, and 1/640.

Erythrocyte Sedimentation Rate (*ESR*).—The Westergren method was used. Blood samples were read after one hour. Only samples which could be set up within 2 hours of collection were included (202 specimens).

Antistreptolysin-O[7] *Antibodies* (*ASO*).—These were titrated by a modified method of Gooder (1961) using defibrinated horse blood. After preliminary screening titrations were performed on all specimens over 50 units.

Antithyroglobulin Antibodies (*TRC*).—The formolized tanned red cell method of Fulthorpe, Roitt, Doniach, and Couchman (1961) was used.[8]

Thyroid Microsomal Antibodies (*CFT*).—The complement-fixation technique of Roitt and Doniach (1958) was used.[9]

Throat and Nose Swabs.—Inocula were cultured overnight in brain-heart infusion broth containing 1:25,000 sodium azide and 1:500,000 crystal violet, and subcultured on blood agar for haemolytic streptococci.

Results

Patients, relatives, and controls are referred to as groups (3), and the groups subdivided by sex as subgroups (6). All means are geometric means unless otherwise stated.

Brucella Antibodies

In the majority of subjects, antibodies to brucella showed a sharp rise one week after injection of antigen (Fig. 1). Levels reached a peak at the end of 2 weeks and gradually declined thereafter, although they were still considerably elevated after 4 weeks. 23 subjects (16 female and 7 male) had brucella antibodies in the serum before immunization and in four cases pre-injection results were not available. Ten subjects had a negative, or minimal, antibody response and of these six had blocking antibodies.

No significant differences[10] in mean responses were observed between the groups, irrespective of whether all participants (Fig. 1B) or only subjects known to be exhibiting a primary antibody response (Fig. 1A) were included in the analysis.

On subdividing the groups according to sex (Fig. 2), all three female subgroups had higher mean antibody titres than the three male subgroups. Thus female patients had a greater mean response than male patients, female relatives than male relatives, and female controls than male controls. For patients, this difference was statistically highly significant ($P < 0·001$) when readings over the test

[6]Agar plates were obtained from Hyland Laboratories.
[7]Streptolysin-O was obtained from Burroughs Wellcome and Co.
[8]Reagents were supplied by Burroughs Wellcome and Co.
[9]Freeze-dried antigen was supplied by Burroughs Wellcome and Co.
[10]Differences not considered significant where $P > 0·05$.

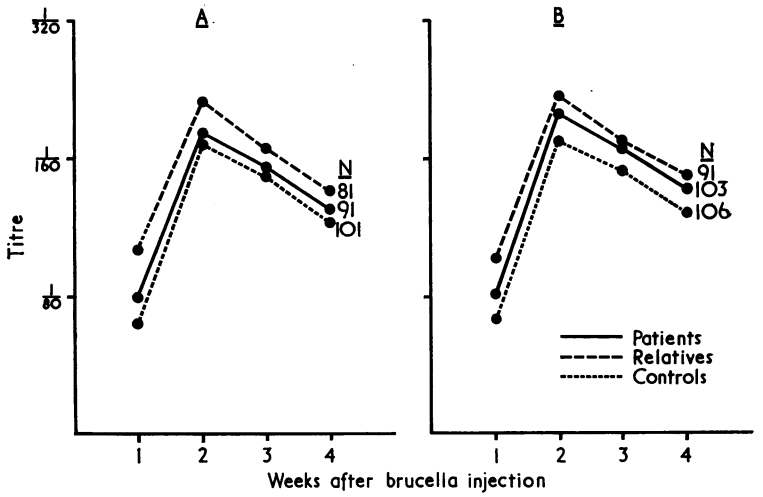

Fig.1.—Antibody response to brucella vaccine. Comparison between patients, relatives, and controls. (A) Primary reactors. (B) All subjects. Differences between groups are not significant for individual weeks nor for all weeks combined.

period were combined (Fig. 2B) and, while the sex difference in antibody levels for relatives and for controls lacked statistical significance, the tendency for higher female levels was clearly present in each case. To avoid a possible bias due to the increased antibody response of known secondary reactors, the majority of whom were females, these and four subjects in whom pre-injection titres were not available were excluded from the analysis, but the exclusion of these individuals did not appreciably alter the statistical significance of the sex differences among patients ($P = 0{\cdot}002$) (Fig. 2A). A comparison between antibody levels of all female and all male primary reactors (Fig. 3A) revealed very significantly higher female titres than male over the test period ($P < 0{\cdot}001$), and females also had significantly raised levels ($P < 0{\cdot}05$) 1, 2, and 3 weeks after antigen injection. Figs 3B and 3C show that this difference was maintained when subjects were subdivided according to the batch of vaccine with which they were immunized (see Fig. 3, overleaf).

Sheep Cell Agglutination Test

Before injection of the antigen, 82 (79 per cent.) patients had a SCAT of 1/32 or over, all controls were negative, and only one relative (1 per cent.) showed a positive result (Table II, overleaf).

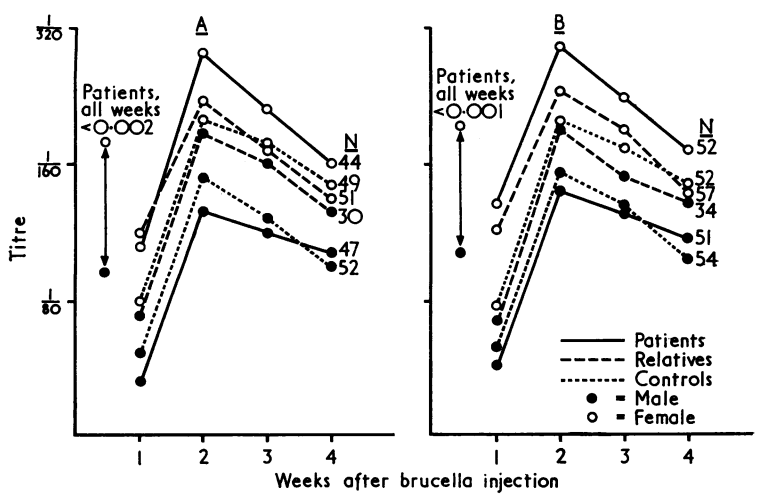

Fig.2.—Antibody response to brucella vaccine. Comparison between the sexes within groups. (A) Primary reactors. (B) All subjects. Differences are not significant except for overall means (all weeks) of men and women patients.

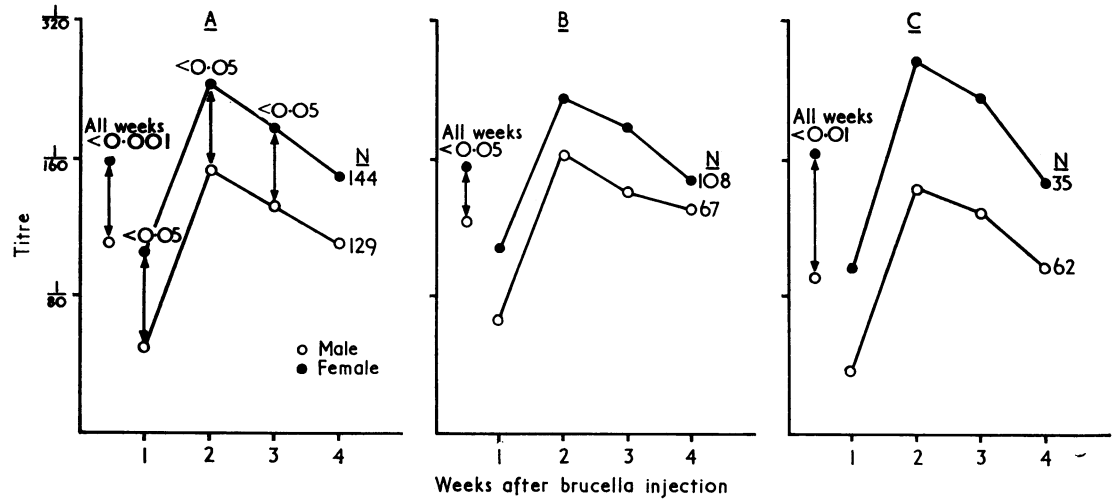

Fig. 3.—Antibody response to brucella vaccine. Comparison of primary responses of all male and female subjects. (A) Vaccine batches I and II. (B) Vaccine batch I. (C) Vaccine batch II. P values of all significant differences are indicated.

TABLE II
RESULTS OF SHEEP CELL AGGLUTINATION AND LATEX SLIDE TESTS, BY SEX, BEFORE AND AFTER BRUCELLA VACCINE INJECTION

Group		No. of Subjects	Sheep Cell Agglutination Test						Latex Slide Test Positive		
			Positive*				Mean‡ Titre (Log_2T)	Significance of Difference between Groups	Before		After
			Before		After†				Before		After
			No.	Per cent.	No.	Per cent.			No.	Per cent.	No.
Patients	Total	103	82	79	7	33	6·89	P < 0·001	87	85	2
	Female ..	52	41	79	4	36			45	87	1
	Male	51	41	80	3	30			42	83	1
Relatives	Total	91	1	1	14	16	2·28		4	4	–
	Female ..	57	0	–	10	17		P < 0·01	2	4	2
	Male	34	1	3	4	12			2	6	–
Controls	Total	106	0	–	3	3	1·88		7	7	–
	Female ..	52	0	–	1	2			1	2	1
	Male	54	0	–	2	4			6	11	–

*Titre of 1/32 or above †Of those initially negative. ‡All five weeks.

Seven patients (33 per cent.) who had initial SCAT titres below 1/32 converted to positive during the period of testing; in one this change was transient on one occasion only, but six remained elevated for 2 or more weeks. Three (3 per cent.) controls became positive, one on one occasion, and two for more than one week. Fourteen (16 per cent.) relatives became positive SCAT, five on only one occasion and nine for 2 or more weeks.

As expected, patients had very significantly higher sheep cell titres than non-patients ($P < 0·001$) and relatives had significantly higher titres than controls ($P < 0·01$) when readings over the test period were combined.

After brucella injection all subgroups showed a rise in sheep cell titre. Patients of both sexes and female relatives exhibited a very significant linear increase in titre ($P < 0·001$) over 4 weeks. Male non-patients showed a lesser rise (but still $P < 0·001$); this was not linear in male relatives and although linear in male controls it was less significant ($P < 0·05$).

Considering all non-patients, instead of relatives and controls separately, linear regression lines were

fitted to both male and female data and a comparison made between their slopes (Fig. 4). Both males and females exhibited a very significant increase ($P < 0.001$), but the rise in SCAT titre for female non-patients was greater than for male, the difference being significant at the level $P = 0.05$; there was no difference between the increases shown by male and female patients.

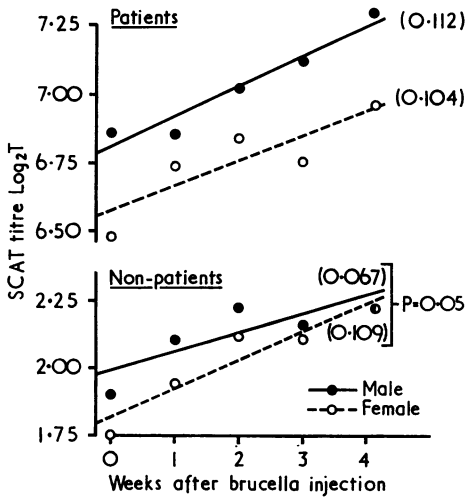

Fig. 4.—Sheep cell agglutination titre increase over 4-week period and comparison of slopes (figures in brackets) of linear regression lines. All increases are significant ($P < 0.001$). Female non-patients show a significantly steeper slope ($P = 0.05$) than male non-patients.

Latex Test

The number of latex positive and negative subjects and of convertors from negative to positive is shown in Table II. There were no significant differences between relatives and controls or between the sexes.

Immunoglobulin M

55 subjects (22 patients, 16 controls, and 17 relatives) had IgM estimations carried out on all five samples of blood. Since over the test period no significant alterations in individual levels occurred, it was decided to carry out IgM estimations on only the first and fourth blood samples of a further 117 subjects. Estimations on sera of all participants were not possible because of the difficulty in obtaining further suitable antiserum.

Mean IgM levels for all patients were very significantly higher than for all non-patients ($P < 0.001$) and there were no differences in values between relatives and controls (Fig. 5, overleaf). Female patients' mean IgM levels were significantly higher than those of male patients ($P < 0.001$) and of female relatives than of male relatives ($P = 0.05$). IgM values for female controls were higher than for male controls but this difference just lacked statistical significance.

The difference between patients and non-patients was mainly due to the significant differences in levels between female patients and non-patients ($P < 0.01$ for female patients and relatives and $P < 0.001$ for female patients and controls). Male patients' mean IgM levels, although slightly higher, did not differ significantly from those of male non-patients. Comparisons of mean levels in male relatives with those in male controls, and in female relatives with female controls, showed no significant differences.

Immunoglobulin G

(*a*) *Modified Rivanol Method.*—Levels determined in five weekly samples of 126 individuals (45 patients, 40 relatives, and 41 controls) showed no significant variations in levels in individuals with time. Estimations of the levels in the sera of the remaining 171 subjects were therefore restricted to two samples, the first and the fourth.

Patients had significantly higher values than controls or relatives ($P < 0.001$) (Fig. 6A, overleaf), and there was no significant difference between the controls and relatives, but female relatives had higher levels than female controls ($P < 0.01$). The only sex difference observed was the significantly increased level ($P < 0.01$) in male controls compared with female controls.

(*b*) *Immunodiffusion Method.*—The second and fifth samples of 176 subjects were tested. Results were broadly comparable with those obtained by the modified rivanol method, but several of the differences found to be significant with (*a*) lacked significance with (*b*) (Fig. 6B) because of the inaccuracy of the latter method. Although patients had higher levels than non-patients, this difference was significant for the women patients only ($P < 0.001$), and the sex difference found by the rivanol method in the controls was not significant by the immunodiffusion method.

Corticosteroid Therapy

Fifteen patients (10 male and 5 female) were receiving oral corticosteroid therapy. The mean values for antibrucella antibodies and immunoglobulin M did not differ significantly in men and women receiving steroids compared with those who were not on this treatment, but IgG levels were significantly lower in patients on steroids ($P < 0.05$).

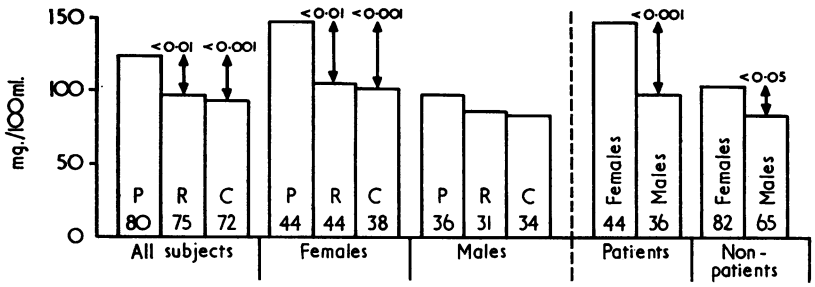

Fig. 5.—IgM mean values. P values of significant differences are indicated. Figures at bases of columns indicate numbers of individuals.

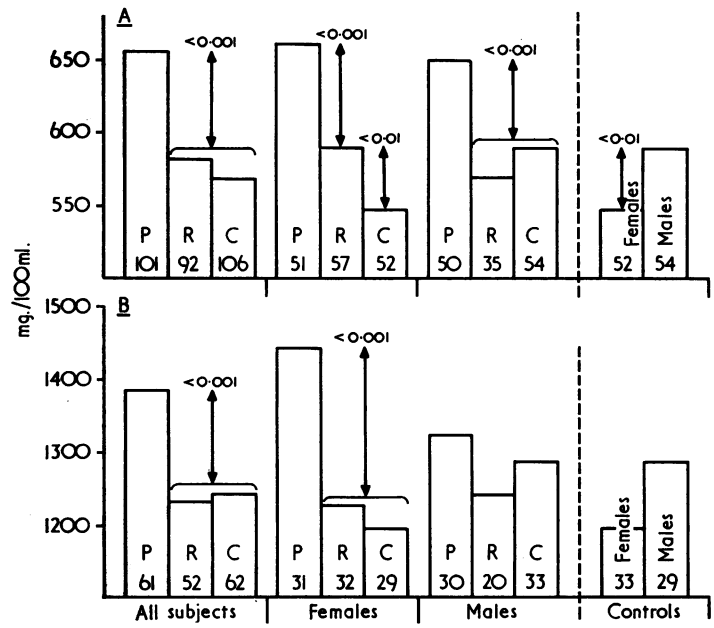

Fig. 6.—IgG mean values. (A) Modified rivanol method. (B) Single radial diffusion. P values of significant differences are indicated. Figures at bases of columns indicate numbers of individuals.

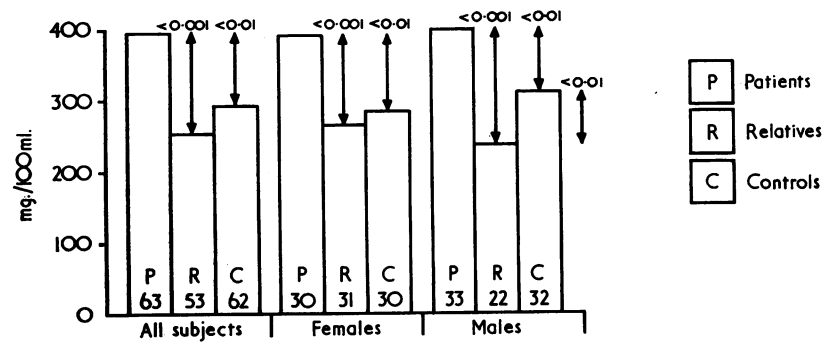

Fig. 7.—IgA mean values. There are no significant differences between the sexes. P values of significant differences are indicated. Figures at bases of columns indicate numbers of individuals.

Immunoglobulin A

The first and fourth samples of each of 180 subjects were tested. The mean level of IgA in patients was significantly greater than in non-patients ($P < 0{\cdot}001$), taking all subjects together or the sexes separately (Fig. 7, opposite). Male and female controls showed a higher mean level than relatives, but the difference was significant only in the case of the man ($P < 0{\cdot}01$). There were no significant differences between the sexes in any of the groups, and levels in individuals appeared to be stable with time.

Antinuclear Factor

All five samples of 163 subjects were tested and the third sample alone of the remaining subjects. No changes in mean titre were detected after brucella injection and titres in individuals were stable. 37 patients (36 per cent.) and three relatives (3 per cent) had a positive ANF; there were no positive controls (Table III).

22 female patients (42 per cent.) but only fifteen male patients (29 per cent.) were positive. Two female relatives (4 per cent.) and one male (3 per cent.) were positive. Although more females than males had antinuclear antibodies, this difference between the sexes was not significant.

Morphologically, the majority of positive tests were homogenous, but two had a speckled and two a peripheral pattern.

Erythrocyte Sedimentation Rate

This was determined in 202 subjects (70 patients, 59 relatives, and 73 controls). The mean (arithmetic mean) initial rate was 30 mm./1st hr. for patients, 8·5 for relatives, and 6·4 for controls. Patients had significantly higher mean values than non-patients ($P < 0{\cdot}001$) and there was no difference between relatives and controls.

There was no rise in the mean rate one week after brucella injection in patients but relatives and controls showed a significant rise ($P < 0{\cdot}001$) (Table IV). Mean rates for female patients were higher than for male patients, but owing to the great variability of the ESR in patients, the sex difference was not significant. Among non-patients females had significantly higher rates than males ($P < 0{\cdot}01$), and this reflects the known elevation of ESR in females, but female non-patients also showed a greater rise in ESR one week after antigen injection

TABLE III
DISTRIBUTION OF ANTINUCLEAR FACTOR

Group	No. of Subjects			Antinuclear Factor Present*					
				Total		Female		Male	
	Total	Female	Male	No.	Per cent.	No.	Per cent.	No.	Per cent.
Patients	103	52	51	37	36	22	42	15	29
Relatives	91	57	34	3	3	2	4	1	3
Controls	106	52	54	0	–	0	–	0	–
Total	300	161	139	40		24		16	

*Positive at dilutions of 1/10 or greater.

TABLE IV
ERYTHROCYTE SEDIMENTATION RATE CHANGES AFTER BRUCELLA VACCINE INJECTION
DIFFERENCES BETWEEN RATES IN MALE AND FEMALE NON-PATIENTS

Group		No. of Subjects	Sample I Mean* (mm./1st hr)	Increase after 1 week (mm./1st hr)	Significance of Increase
Relatives		59	7·4	1·7	$P < 0{\cdot}001$
Controls		73	6·0	1·4	$P < 0{\cdot}001$
Relatives and Controls	Female	79	7·7	1·8	$P < 0{\cdot}001$
	Male	53	5·0	1·2	$P < 0{\cdot}05$
			Overall Mean*	Significance of Sex Difference	
Relatives and Controls	Female	79	8·7	$P < 0{\cdot}01$	
	Male	53	5·5		

*Arithmetic mean.

($P < 0{\cdot}001$) than male non-patients in whom the rise was much less significant ($P < 0{\cdot}05$).

Antistreptolysin-O Antibodies

ASO titres showed no significant change with time. No differences were found in the proportions of patients and non-patients, or of men and women, having high and low ASO titres ($\geqslant 150$ and < 150 units).

Thyroid Antibodies

These were tested in 95 cases (33 patients, 38 relatives, and 24 controls) on five blood samples. The TRC was positive (greater than 1/250) in two (6 per cent.) patients, one of whom was known to have thyroid disease, one (3 per cent.) relative, and one (4 per cent.) control. The complement-fixation test was positive in one relative only. These tests were discontinued for the remaining subjects because the incidence of positive tests was no greater than could be expected in the general population.

Streptococci

Haemolytic streptococci belonging to Lancefield Groups A, C, or G were not isolated from any nose or throat swabs.

Venereal Disease

Serological tests for syphilis were negative in all subjects.

Discussion

Our findings suggest the presence of fundamental immunological differences between men and women mainly concerning the macroglobulin system. We observed no differences in antibody response to brucella between rheumatoid patients, their relatives, and controls, and this is in agreement with the findings of Shearn, Epstein, and Engleman (1963) and of Waller, Ellman, and Toone (1966), and with results of others, who used different antigens (Larson and Tomlinson, 1951; Creger, Choy, and Rantz, 1951; Barr, Buchanan, Doniach, and Roitt, 1964). Conflicting findings of heightened antibody response in rheumatoid patients were reported by Greenwood and Barr (1960), Meiselas, Zingale, Lee, Richman, and Siegel (1961), and Houba, Adam, Malecek, and Tesarek (1964). The sex distribution was not published in the above investigations, except by Barr and others (1964), who used patient groups which were predominantly, and control groups which were entirely, female, and by Meiselas and others (1961), who compared female patients with male controls. These authors interpreted the increased immunological response of patients over controls as evidence of hyperreactivity, whereas their observations may have reflected differences in reactivity between men and women.

Our findings confirm the results of recent studies of normal populations which reveal higher IgM levels in women in the United States (Lichtman, Vaughan, and Hames, 1967; Butterworth, McClellan, and Allansmith, 1967) and in Denmark (Jensen, 1967). Studies which show that American Negroes have higher immunoglobulin levels than Caucasians (Pollak, Mandema, Doig, Moore, and Kark, 1961; Fudenberg, 1963), Tibetans than Swiss (Hartmann and Ritzel, 1967), and African Negroes domiciled in England than Caucasians (Cohen, McGregor, and Carrington, 1961) provide further evidence that immunoglobulin levels are under genetic control.

In this study a heightened sensitivity of the normal female immune system compared with that of the male is also revealed, after brucella vaccine injection, by a higher specific antibody response and a greater anamnestic response. Evidence for the latter lies in the significantly greater rise in SCAT titres, and the increased immunological reactivity is reflected in a steeper rise in the ESR. It has been shown that the immediate antibody to brucella is usually an IgM (Reddin, Anderson, Jenness, and Spink, 1965; Kerr, Payne, Robertson, and Coombs, 1967); further studies will reveal whether this greater response in females is induced by other antigens.

The idea of a hyperreactive host response in females receives support from experimental work on animals concerned with homograft rejection (Brent and Medawar, 1966), with host susceptibility to bacteria (Wheater and Hurst, 1961), tumour cells (Gorer and Kaliss, 1959; Batchelor and Chapman, 1965), and foreign proteins (Dresser, 1962), and with immune depression after neonatal thymectomy (Balner and Dersjant, 1966).

If antibodies were produced only after antigenic stimulation, one might expect that a hyperreactive diathesis in females would not become manifest until after antigenic challenge, in late infancy and particularly during school years. In this context, it is worthy of note that Butterworth and others (1967) reported significantly higher IgM levels in girls over the age of 7 compared with boys, while others observed no such differences in early infancy (Stiehm and Fudenberg, 1966). Support for a more sensitive host response in females also comes from reports which indicate that the mortality rates of female infants from infections are significantly

lower than those of male infants. Immunological deficiency diseases such as congenital agammaglobulinanaemia are commoner in male infants (Janeway, 1966a, b), suggesting greater vulnerability of the male immune system.

We found no significant individual variation in serum concentrations of immunoglobulins G, A, or M during the time of the study, and it appears that these immunoglobulins are normally stable for each individual (Allansmith, McClellan, and Butterworth, 1967). We cannot explain the higher levels of IgG in male than in female controls ($P < 0{\cdot}01$), and more work is necessary to confirm these results.

Sex hormones, corticosteroid therapy, and chronic infection may alter immune responses. Chronic infection was ruled out in our controls by strict selection and the relatives were also free from clinically obvious disease. The effect of sex hormones is known from animal experiments wherein oestrogens tend to stimulate the reticuloendothelial system while testosterone may depress immunological activity (von Haam and Rosenfeld, 1942; Sherman, Adner, and Dameshek, 1963). As 49 per cent. of our female participants were over the age of 50 it appeared improbable that oestrogens would have caused the higher level of circulating antibodies in women than in men, but it was thought possible that male hormones may have depressed antibody production in the men. However, the younger subjects (up to 50 years of age) of each sex produced significantly more antibodies than the older members of the same sex, and this difference was much less significant for the women ($P < 0{\cdot}05$) than for the men ($P < 0{\cdot}001$) (Fig. 8). Considering these results, it is unlikely that the differences in antibody levels were sex hormone dependent.

Corticosteroids are known to depress the reticuloendothelial system. However, the mean primary response antibrucella antibody levels of eight males and four females on steroid therapy proved not to be significantly different from the antibody levels of the remaining patients of corresponding sex.

The above considerations are also relevant to IgM levels. Fig. 8 shows significantly higher mean IgM levels in younger, compared with older, normal women ($P < 0{\cdot}001$). This, in the light of the experimental work mentioned, would suggest oestrone-dependent increase of IgM production, particularly as no change with age is present in normal men. However, the issue is not clear-cut, as male, but not female, rheumatoid patients show a significant age difference in IgM levels, values being higher in younger male patients compared with those over 51 years of age ($P < 0{\cdot}025$), but there is no difference in mean levels between younger and older female patients.

Reports of immunoglobulin levels in rheumatoid arthritis are variable (Barden, Mullinax, and Waller, 1967; Bakker, Imhof, Mul, and Ballieux, 1967); Wasastjerna, Vilppula, and Jeglinsky, 1967; Claman and Merrill, 1966) and the possibility of sex differences was not explored in these studies. In this investigation, immunoglobulins G, A, and M were significantly increased in all patients compared with controls or relatives, but differences in levels between the sexes were present with IgM

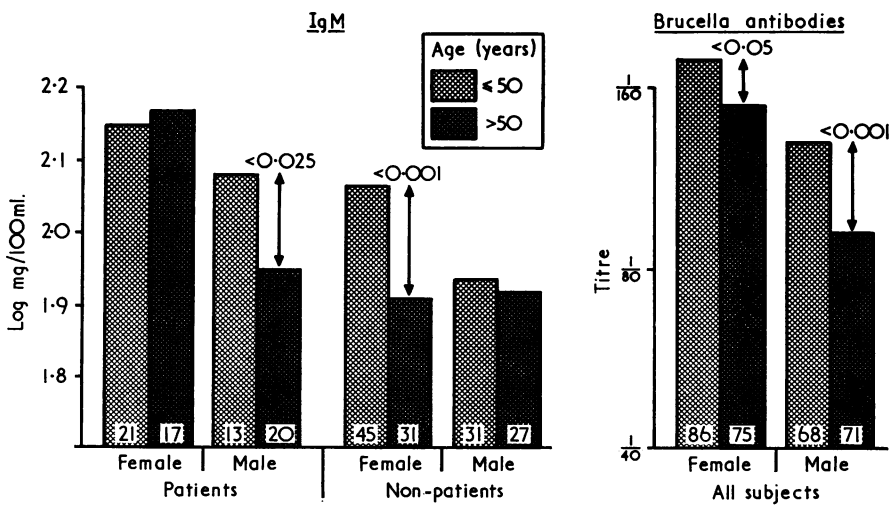

Fig. 8.—Mean levels of IgM and of anti-brucella antibodies in younger subjects (50 years and less) and older subjects (over 50 years). P values of significant differences are indicated. Figures at bases of columns indicate numbers of individuals.

only ($P < 0{\cdot}001$), female patients having higher values than male patients. Rheumatoid men did not have significantly higher values than non-rheumatoid men, and the high P value obtained for the IgM difference between patients and controls ($P < 0{\cdot}001$) was due mainly to higher values in women patients.

Female patients also had a higher incidence (not statistically significant) of antinuclear antibodies (42 per cent.) compared with male patients (29 per cent.). Other workers have observed an increased incidence of antinuclear antibodies in women (Seligmann, Cannat, and Hamard, 1965; Svec and Veit, 1967). Under experimental conditions female animals likewise seem to exhibit autoimmune phenomena more readily than male animals (Pearson, 1959; Bielschowsky, Helyer, and Howie, 1959; Helyer and Howie, 1961; Holborow and Denman, 1967).

In our subjects the incidence of rheumatoid factor was equal for the sexes. This is in agreement with findings of Ball and Lawrence (1961) in multiple population samples involving several European countries, and of Litwin and Singer (1965) in aged people in New York. There is little doubt that the small, but statistically highly significant, rise in mean sheep cell titres ($P < 0{\cdot}001$) in all groups was due to brucella vaccine injections. A slight increase in sensitized sheep cell agglutinins associated with antigenic stimulation can be expected, as conversion to sero-positivity has been reported in diverse infective conditions (Bonomo, Gillardi, and Tursi, 1966; Williams and Kunkel, 1962; Singer, Plotz, Peralta, and Lyons, 1962; Mustakallio, Lassus, and Wager, 1967). The failure of Waller and others (1966) to detect changes in rheumatoid factor titres after brucella injection may have been due to their small sample size. In patients, we did not detect the same sex differences in SCAT rise as were present in non-patients (Fig. 4), but this may have been due to the much greater variation of titres encountered among patients.

The question arises whether immunoglobulin M levels in rheumatoid women are increased as a result of the disease or whether previously elevated levels are related to the greater female incidence of autoimmune disease. Negroid races, in whom immunoglobulin G levels are high, apparently as a result of hereditary influence, also have a much increased incidence of such connective tissue diseases as sarcoid (Hunt, 1966) and systemic lupus erythematosus (Siegel and Seelenfreund, 1965). The ability of normal females, intensified in females with rheumatoid arthritis, to effect higher levels and more rapid mobilization of antibodies than males raises the question of an aetiological link between this hyperreactivity and the female predisposition to rheumatoid disease. Might a more sensitive immune system perhaps have conferred phylogenetic advantages on the female, these advantages, however, tending to be offset by an increased susceptibility to connective tissue disease? But were a heightened immune response an invariably necessary predisposing factor it might be expected that the disease would be found among that section of males having such a heightened response, and the sex differences would then be less in patients than in normal persons. Inasmuch as the differences that are present between the sexes in normal individuals are retained and even intensified in patients with rheumatoid arthritis, it is clear that the relationship between the sex differences and the sex incidence of the disease is a complex one.

Summary

Antibody responses to brucella vaccine were studied in 300 individuals, of whom 103 had rheumatoid arthritis, 91 were healthy relatives of the patients, and 106 were matched controls. The proportions of male and female subjects were almost equal.

Female patients, relatives, and controls all produced higher mean levels of specific antibody to brucella than the corresponding males, and the sex difference in antibody response was statistically highly significant. No differences in antibody production were observed between patients, relatives, and controls.

After an injection of brucella vaccine, sheep cell agglutination tests for rheumatoid factor showed significant increases in mean titres in all groups. Patients of both sexes and female relatives and controls had a more significant increase than male relatives and controls, and this difference between male and female non-patients was statistically significant.

Mean levels of immunoglobulin M were significantly higher in all female subjects compared with males, and female patients had the highest levels. Serum concentrations were significantly higher in female patients than in female non-patients, but in males values did not differ significantly in patients and non-patients. No changes in IgM were detected after brucella injection compared with pre-injection levels.

No significant sex differences were observed in IgG and IgA levels. Patients had significantly higher levels than non-patients. Levels in individuals were stable during the test period.

The occurrence of antinuclear antibodies was more frequent in female patients than in males, but the difference was not significant.

Mean erythrocyte sedimentation rates showed a greater rise in female non-patients than in males. Patients' mean sedimentation rates remained unchanged.

No significant increases were found in patients compared with non-patients in the levels of thyroid, or antistreptolysin-O, antibodies.

These findings of sex differences in immunological responsiveness are discussed in relation to the increased incidence of rheumatoid disease in females.

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APPENDIX

Statistical Methods

The analysis of variance was used to separate the very considerable differences between subjects from the much smaller changes shown by individuals from week to week. As an example, the analysis of the brucella figures is given below and any differences from this design are referred to under the separate investigations.

Brucella Antibodies.—The antibody response was measured by the last titre (T) to show agglutination, the dilutions being 20, 40, 80 times, etc. Absence of agglutination at 1 in 20 was taken as zero. The figures used in the calculations were $\log_2(T/10)$, *i.e.* 1, 2, 3, etc. The means calculated were therefore mean logs; the antilogs are the geometric means of the titres, which are always less than arithmetic means. Table V shows the weekly totals for the six sub-groups and Table VI the analysis of variance. The residual mean square for testing between subjects was 8·51, between weeks 0·646. The standard errors of the sub-group means for testing the differences between the sexes shown in Table V are $\sqrt{8 \cdot 51/4 \times \text{number in sub-group}}$; the standard errors of the weekly sub-group means for testing changes from week to week are $\sqrt{0 \cdot 646/\text{number in sub-group}}$. Since there were different proportions of men and women in the three groups a correction for disproportion was made in the analysis (Snedecor, 1956).

SCAT.—The figures used were $\log_2$ T, where T is the last titre to show agglutination. Since patients as a group have very much higher titres than non-patients, and correspondingly big variances, they were analysed separately from non-patients. Straight lines could be fitted to the weekly means for each sub-group. In the analysis that part of the "between weeks" sum of squares ascribable to linear regression is shown. As an example the analysis of the figures for women non-patients is given (Table VII).

TABLE V
BRUCELLA ANTIBODIES
Weekly Totals in Six Subgroups

Group	Patients		Relatives		Controls		Total	
Subgroup	Male	Female	Male	Female	Male	Female	Male	Female
No. of Subjects	51	52	34	57	54	52	139	161
Week 2	128	184	97	201	144	154	369	539
Week 3	194	253	145	259	212	224	551	736
Week 4	186	233	133	242	198	214	517	689
Week 5	176	213	126	216	178	200	480	629
Totals	684	883	501	918	732	792	1917	2593
Means	3·353	4·245	3·684	4·026	3·389	3·808	3·448	4·026
Sex Difference	0·892***		0·342		0·419		0·578***	

***P < 0·001 **P < 0·01 *P < 0·05

TABLE VI
BRUCELLA ANTIBODIES
Analysis of Variance

Variation	Source of Variation	Sums of Squares	Degrees of Freedom	Mean Square
Between 300 Subjects	Sex (S)	93·07	1	93·07***
	Groups (G)	12·56	2	6·28
	Interaction (S × G)	17·51	2	8·75
	Residual	2501·78	294	8·51
	Total	2624·92	299	
Within Subjects	Weeks (W)	267·09	3	89·03***
	Interaction (W × subG)	10·29	15	0·686
	Residual	569·62	882	0·646
	Total	847·00	900	
Grand Total		3471·92	1199	

IgM.—The figures analysed were $\log_{10} g/10$, where g was the concentration assuming the standard to contain 100 units of antigen. The antilogs of the mean logs were finally converted to mg/100 ml. by references to a solution of antigen of known strength. Estimations for all the six sub-groups for weeks 1 and 4 were analysed together, as for brucella.

IgG.—Concentrations in cg./100 ml. were used. A separate analysis was carried out for patients, because their residual variance (between subjects) was approximately twice that for non-patients.

IgA.—Concentrations in cg./100 ml. were the figures used. The three groups were analysed separately because their residual mean squares (between subjects) differed significantly. The appropriate test was used in testing the differences between the group means.

ESR.—Again the patients were analysed separately, their residual variance being about twelve times that of non-patients.

ASO.—The χ^2 test was used to test the difference between the proportions in the three groups having high ($\geqslant 150$) and low (< 150) ASO titres, and also the difference in these proportions between the two sexes.

TABLE VII
SCAT WEEKLY TOTALS FOR FEMALE NON-PATIENTS, WITH ANALYSIS OF VARIANCE

Group	No. of Subjects	Week 1	2	3	4	5
Relatives	57	110	120	131	130	138
Controls	52	80	92	99	99	103
Total	109	190	212	230	229	241
Means		1·743	1·945	2·110	2·101	2·211

Variation	Source of Variation	Sums of Squares	Degrees of Freedom	Mean Square
Between 109 Women	Subgroups (subG)	20·45	1	20·45*
	Residual	458·89	107	4·29
	Total	479·34	108	
Within Women	Linear regression	12·99	1	12·99***
	Departures	1·56	3	0·52
	Between weeks W	14·55	4	3·64***
	Interaction (W × subG)	0·15	4	0·04
	Residual	149·70	428	0·350
	Total	164·40	436	
	Grand Total	643·74	544	

$$\text{Overall Mean Rise} = 2{\cdot}211 - 1{\cdot}734 = 0{\cdot}468$$

$$\text{"}t\text{"} = \frac{0{\cdot}468}{\sqrt{2 \times 0{\cdot}350/109}} = 5{\cdot}84^{***}$$

*P < 0·05 ***P < 0·001

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Les différences immunologiques d'après le sexe du malade. Une étude des malades atteints de polyarthrite rhumatoïde, de leurs parents et des témoins

RÉSUMÉ

La formation d'anticorps contre le vaccin de la brucellose a été étudiée chez 300 personnes; 103 étaient atteintes de polyarthrite rhumatoïde, 91 étaient des parents sains de ces malades et 106 étaient des témoins assortis. Les proportions d'hommes et de femmes étaient à peu près égales.

Les femmes malades, leurs parents et les témoins, tous avaient produit, en moyenne, des taux plus élevés de l'anticorps spécifique à la brucellose que le groupe d'hommes correspondant, la formation d'anticorps due au sexe était très significative. Aucune différence dans la production d'anticorps n'avait été observée entre les malades, leurs parents et les témoins.

Après une injection de vaccin contre la brucellose, les tests d'agglutination aux cellules du mouton pour le facteur rhumatoïde avaient montré des augmentations significatives dans la moyenne des titres de tous les groupes. Les malades des deux sexes, les parents et les témoins du sexe féminin avaient une augmentation plus marquée que les parents et les témoins masculins, et cette différence statistique entre les témoins des deux sexes était significative.

Diferencias inmunológicas entre los sexos. Un estudio de pacientes con poliartritis reumatoide, sus parientes y testigos

SUMARIO

Las reacciones de anticuerpos a la vacuna brucella fueron estudiadas en 300 individuos, de los cuales 103 padecían poliartritis reumatoide, 91 eran parientes saludables de los pacientes, y 106 testigos iguales. Las proporciones de sujetos masculinos y femeninos eran casi iguales. Los pacientes, familiares y testigos femeninos produjeron niveles más altos de anticuerpos específicos contra la brucella, que los correspondientes masculinos, y la diferencia de sexo en la reacción de anticuerpos fue, estadísticamente, muy significativa. No se observó ninguna diferencia en la producción de anticuerpos entre pacientes, parientes y testigos.

Después de una inyección de vacuna brucella, las pruebas de aglutinación de célula de oveja en busca de factores reumatoides mostraron aumentos significativos en las concentraciones promedio en todos los grupos. Los pacientes de ambos sexos y los parientes y testigos femeninos tuvieron un aumento más significativo que los parientes y testigos masculinos, y esta diferencia entre hombres y mujeres no pacientes fue estadísticamente significativa.

La moyenne des taux d'immunoglobuline M était plus marquée chez tous les sujets du sexe féminin comparée à celle des sujets mâles et les patientes avaient les plus hauts taux. Les concentrations de sérum étaient beaucoup plus élevées chez les patientes que chez les parentes et les témoins, mais chez les hommes les valeurs ne différaient pas beaucoup chez les patients et les sujets sains. Aucun changement dans l'IgM avait été remarqué après l'injection du vaccin contre la brucellose comparé aux taux obtenus avant l'injection.

Aucune différence marquée due au sexe n'avait été observée dans les taux IgG et IgA. Les patients avaient des taux plus élevés que les sujets sains. Les taux individuels étaient fixes pendant la période de l'expérience.

L'apparition des anticorps antinucléaires était plus fréquente chez les patientes que chez les patients, mais la différence n'était pas significative.

La moyenne des taux de sédimentation des érythrocytes avait montré une hausse plus grande chez les femmes saines que chez les hommes.

Aucune hausse significative n'avait été trouvée chez les malades comparée aux sujets sains dans les taux d'anticorps de l'antistréptolysine-O ou de la thyroide.

Ces différences de la réponse immunologique dues au sexe sont discutées en relation à l'incidence accrue de la maladie rhumatoïde chez les femmes.

Los niveles promedios de inmunoglobulina M fueron notablemente más altos en todos los sujetos femeninos comparados con los masculinos, y los pacientes femeninos tuvieron los niveles más elevados. Las concentraciones de suero fueron significativamente más altas en pacientes femeninos que en mujeres no pacientes, pero en los masculinos los valores no diferían significativamente en pacientes y no pacientes. No se notaron cambios en IgM después de la inyección de brucella, en comparación con los niveles antes de la inyección.

No se observaron diferencias significativas de sexo en los niveles de IgG y IgA. Los pacientes acusaban niveles significativamente más altos que los no pacientes. Los niveles individuales eran estables durante el período de prueba.

La aparición de anticuerpos antinucleares fue más frecuente en los pacientes femeninos que en los masculinos, pero la diferencia no fue significativa.

Las tasas promedio de sedimentacion de eritrocitos mostraron un incremento mayor en los no pacientes femeninos que en los masculinos.

No se encontró ningún aumento significativo en los pacientes, comparados con los no pacientes, en los niveles de anticuerpos de la tiroides, o antistreptolisin-O.

Estas diferencias entre los sexos, con respecto a la reacción inmunológica, son discutidos en relación a la creciente incidencia de la enfermedad reumatoide en las mujeres.