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MEASUREMENT OF ANTIBODY-PRODUCING CAPACITY TO FLAGELLIN IN MAN

IV. STUDIES IN AUTOIMMUNE DISEASE, ALLERGY AND AFTER AZATHIOPRINE TREATMENT*

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SUMMARY

Humoral immune responses after primary and secondary immunization with monomeric flagellin from *Salmonella adelaide* were examined over 10 weeks in three groups of patients with putative autoimmune disease, i.e. systemic lupus erythematosus, rheumatoid arthritis and lupoid hepatitis, in patients with allergy to an extrinsic antigen, and in azathioprine treated patients. Sera were titrated by tanned cell haemagglutination for total antibody and IgG antibody.

In patients with autoimmune diseases and allergy the humoral immune response to flagellin was similar to that of matched controls with other illnesses, so that neither autoimmunity nor allergy could be attributed to any general 'overactivity' of the antibody producing system.

In patients with autoimmune diseases treated with azathioprine there was no statistically significant depression of the primary or secondary humoral immune response to flagellin, as compared with appropriate controls. This could be explained by azathioprine having a predominantly anti-inflammatory action, influencing cellular more than humoral immune mechanisms, in being 'tolerogenic' in respect of autoantigens, or by a combination of these effects.

INTRODUCTION

This study was undertaken to ascertain whether autoimmune diseases and asthma were associated with a demonstrable general hyperreactivity of the immunological system, and to assess the immunosuppressive effect of azathioprine as used in the treatment of autoimmune disease. Antibody-producing capacity to the antigen flagellin was tested in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis, lupoid hepatitis, allergic

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(atopic) disease, and in patients treated with azathioprine. For most groups both primary and secondary immune responses were measured.

Our findings indicated that patients with the above diseases gave responses equivalent to those of matched controls in hospital. The indications were that autoimmune disease and allergy are not associated with immunological hyperactivity, and that azathioprine in conventional doses has therapeutic effects which seem independent of suppression of the humoral immune response to an extrinsic antigen.

MATERIALS AND METHODS

Immunization and titration

Monomeric flagellin from *Salmonella adelaide* was prepared as described by Ada *et al.* (1964), passed through a sterile Seitz filter (No. 9 pad) and stored at −20°C until use. Subsequent handling of the antigen was as described previously (Rowley & Mackay, 1969; Lee, Rowley & Mackay, 1970). Samples of blood were obtained before injection and thereafter at 6–8 days ('1 week'), 14–21 days ('2 weeks'), 35–49 days ('6 weeks') and 63–77 days ('10 weeks').

TABLE 1. Numbers of cases, age and sex of patients studied

Clinical group		Total No.	Sex		Age (years)	
			M	F	Mean	Range
Systemic lupus erythematosus	I*	16	4	12	36	16–74
	II†	17	6	11	36	18–61
Rheumatoid arthritis	I*	20	10	10	48	23–67
	II†	6	5	1	50	36–69
Active chronic hepatitis		12	5	7	47	16–66
Allergy		16	6	10	44	19–68
Azathioprine treated	I*	20	4	16	35	12–59
	II†	11	2	9	40	16–68

I* = primary response; II† = secondary response.

Titration of antibody to flagellin

Titrations were done by tanned sheep red cell haemagglutination, using cells coated with polymerized flagellin (Wistar, 1968), for both total antibody and antibody remaining after treatment of serum with 2-mercaptoethanol (ME) for 1 hr at 37°C. ME-sensitive antibody was shown by gel filtration of serum through a Sephadex G-200 column to be present in the IgM peak and ME-resistant antibody in the IgG peak (Rowley, Wistar & Mackay, 1971).

Patients and diagnoses (Table 1)

Systemic lupus erythematosus (SLE) (sixteen patients). The diagnosis was made according to the criteria of Dubois (1966). Four of sixteen patients tested for the primary response were in relapse and were receiving 30–60 mg of prednisolone daily (mean 41 mg), and twelve

had quiescent disease with prednisolone doses of 5–25 mg daily (mean 16 mg) in ten, and nil in two cases. The secondary response was tested in seventeen patients who were receiving 7–60 mg daily of prednisolone (mean 17·5 mg), and were reinjected with flagellin at a mean time of 12 months after the first injection.

Rheumatoid arthritis (*twenty patients*). The diagnostic criteria were those of the American Rheumatism Association (Ropes *et al.*, 1956); fourteen were sero-positive for rheumatoid factor by the sensitized sheep cell agglutination method. Four of twenty patients tested for the primary response were receiving prednisolone, 5–12·5 mg daily, or ACTH, 20 units daily, and the others salicylates, indomethacin or phenyl butazone. The secondary response was tested in six patients of whom three were receiving prednisolone, 5–15 mg daily.

Lupoid hepatitis (*twelve patients*). The diagnostic criteria were those of Mackay, Weiden & Hasker (1965). Three patients were receiving prednisolone, 3–20 mg daily, and the remainder with quiescent disease were receiving no treatment; those receiving azathioprine were excluded. The secondary response was not tested.

Allergy (*sixteen patients*). These patients had hay-fever (eleven cases) and/or asthma (five cases) with perennial exacerbations and a positive immediate cutaneous sensitivity reaction to spring grass pollen. Four asthmatics were receiving prednisolone, 5–15 mg daily, and those with hay-fever were receiving anti-histamine preparations. The secondary response was not tested.

Azathioprine-treated patients (*twenty patients*). The primary response was tested in patients with lupoid hepatitis (eleven), SLE (three), Sjögren's disease (two), chronic iridocyclitis (two), corneal graft rejection (one) and ulcerative colitis (one). The dose of azathioprine ranged from 100 to 200 mg daily (mean 150 mg) and thirteen patients were also receiving prednisolone, 5–35 mg daily (mean 13 mg). All patients were immunized at least 3 months after starting treatment which was continued throughout the 10-week period of testing.

Secondary responses were tested in eleven patients including those with lupoid hepatitis (six), SLE (two), chronic iridocyclitis (one), corneal graft rejection (one) and ulcerative colitis (one). The dose of azathioprine ranged from 50 to 200 mg daily (mean 145 mg) and seven patients were receiving also prednisolone, 5–15 mg daily (mean 7·5 mg). The patients were reinjected with flagellin at a mean time of 12 months after the first injection, and azathioprine was given throughout the period between the two injections.

Controls

The controls for the various groups of patients tested for primary and secondary responses to flagellin were derived from patients in hospital suffering from miscellaneous non-neoplastic diseases not known to affect the immunological system. Each group of patients was compared with a randomly selected but age and sex matched group of hospital controls which was double the number of patients. The group with allergy was compared also with a similarly matched group of healthy controls. The twenty controls for the primary response of azathioprine-treated patients were matched for age and sex and were suffering from similar illnesses, i.e. SLE (thirteen cases) and lupoid hepatitis (seven cases), but were not receiving azathioprine; thirteen were receiving comparable amounts of prednisolone, 3–55 mg daily (mean 20 mg). The twenty-two controls for the secondary responses of azathioprine-treated patients were double in number of tested subjects (eleven), and were age and sex matched. Their diagnoses included SLE (twelve), rheumatoid arthritis (three), lupoid hepatitis (three), pernicious anaemia (two), chronic thyroiditis (one) and discoid

TABLE 2. Geometric mean titres and range (±2 S.E.) for total and IgG antibody before and 2 and 10 weeks after primary injection of flagellin

Time	Antibody	Systemic lupus erythematosus		Rheumatoid arthritis		Active chronic hepatitis		Allergy		Azathioprine-treated	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Pre-immunization	Total	13 (39)	5·65 29·9 (20·1–75·7)	18 (33)	7·1–45·6 (18·2–59·8)	69 (29)	20·0–238 (13·6–61·7)	44 (38) *42*	22·8–85·0 (20·9–69·2) *17·3–102*	52 (28)	19·2–141 (11·2–70)
	IgG	1 (1)	1–1·61 (1–1·22)	1 (1)	1–1·48 (1–1·28)	1 (1)	1–1·44 (1–1·21)	1 (1) *1*	1–1·38 (1–1·26) *1–1·56*	1 (1)	1–1·45 (1–1·41)
After 2 weeks	Total	1840 (2540)	502–6750 (1250–5140)	1990 (775)	767–5210 (346–1740)	1720 (782)	734–4030 (279–2190)	4180* (1100) *6230*	1650–10600 (532–2260) *2990–12960*	950 (1900)	355–2550 (778–4640)
	IgG	147 (57)	28·4–761 (19·8–164)	87 (37)	23·9–317 (13·4–102)	83 (55)	17·4–396 (14·6–209)	430† (37) *168*	91·1–1780 (13·5–101) *49·6 –569*	38 (85)	11·1–131 (20·4–354)
After 10 weeks	Total	215 (555)	83·6–553 (315–978)	267 (279)	97·8–729 (130–599)	159 (335)	46·1–549 (150–750)	673 (324) *1510*	333–1360 (171–615 *87·5–2590*	381 (255)	79·8–790 (108–601)
	IgG	6 (22)	1·41–25·5 (9·26–52·3)	20 (15)	6·01–66·5 (5·76–38·9)	7 (45)	1–50·2 (15·5–131)	29 (18) *7*	7·49–112 (7·5–43·2) *1·89–25·9*	9 (9)	3·05–26·6 (2·29–35·3)

All data are given to three significant figures. Mean titres for hospital controls are shown in parentheses and for healthy controls in italics.
* $0{\cdot}01 < P < 0{\cdot}05$. † $P = 0{\cdot}01$.

lupus erythematosus (one); eleven were receiving prednisolone, 7–60 mg daily (mean 17·5 mg). The average time interval between the primary and secondary immunization in these controls was 13 months.

The influence of prednisolone on the immune response was assessed by selecting, from patients with either SLE, rheumatoid arthritis or lupoid hepatitis, a prednisolone treated and a non prednisolone treated group each of fourteen patients, ten females and four males. The mean peak response to flagellin of the two groups was compared.

RESULTS

The findings are set out in Tables 2 and 3 and Figs 1–5.

Natural antibody to flagellin

Natural antibody to flagellin of IgM class can be detected in most individuals before immunization with flagellin (Rowley & Mackay, 1969; Rowley, 1970). The incidence of

TABLE 3. Geometric mean titres and range (±2 S.E.) for total and IgG antibody before and 2 and 10 weeks after secondary injection of flagellin

		Systemic lupus erythematosus		Rheumatoid arthritis		Azathioprine-treated	
Time	Antibody	Mean	Range	Mean	Range	Mean	Range
Pre-immunization	Total	71 (122)	33·1–153 (73·5–203)	89 (94)	26·0–304 (31·9–277)	189 (66)	56·0–638 (34·2–128)
	IgG	12 (8)	4·56–31·6 (4·32–14·8)	18 (7)	3·22–101 (2·20–22·2)	30 (9)	5·9–121 (4·1–19·8)
After 2 weeks	Total	4140 (4640)	1550–11100 (2330–9260)	12600 (6840)	3810–41400 (3050–15300)	4160 (4780)	1550–11100 (2250–10200)
	IgG	852 (1640)	226–3210 (590–4560)	4450 (4830)	995–19900 (1690–13800)	2000 (1100)	569–7040 (355–3420)
After 10 weeks	Total	802 (2180)	247–2610 (1020–4660)	1730 (5310)	572–5220 (1860–15200)	2990 (1300)	589–15200 (521–3230)
	IgG	240 (773)	46·8–1230 (238–2510)	655 (3110)	191–2240 (773–12500)	812 (441)	87–7580 (135–1440)

All data are given to three significant figures.
Mean titres for hospital controls are shown in parentheses.

natural antibody in the disease groups and in matched controls (in parentheses) was for SLE 88% (93%), rheumatoid arthritis 85% (92%), active chronic hepatitis 92% (91%), allergy 100% (94%) and azathioprine treatment 80% (85%); these differences were not significant. Mean titres of natural antibody were lower, but not significantly so, than those for controls in groups with SLE and rheumatoid arthritis (Table 2).

Immune responses to flagellin

Systemic lupus erythematosus. The mean peak titre of total and IgG antibody after 2

weeks was similar to that of the controls, although at 10 weeks the mean titres of total and IgG antibody (215 and 6) were lower than in controls (555 and 22) (Fig. 1a). The secondary response was similar in the patients and controls (Fig. 1b).

Rheumatoid arthritis. The mean peak titre of total and IgG antibody in the primary response was slightly but not significantly greater than that of the hospital controls (Fig. 2a); the secondary response in patients and controls was similar (Fig. 2b).

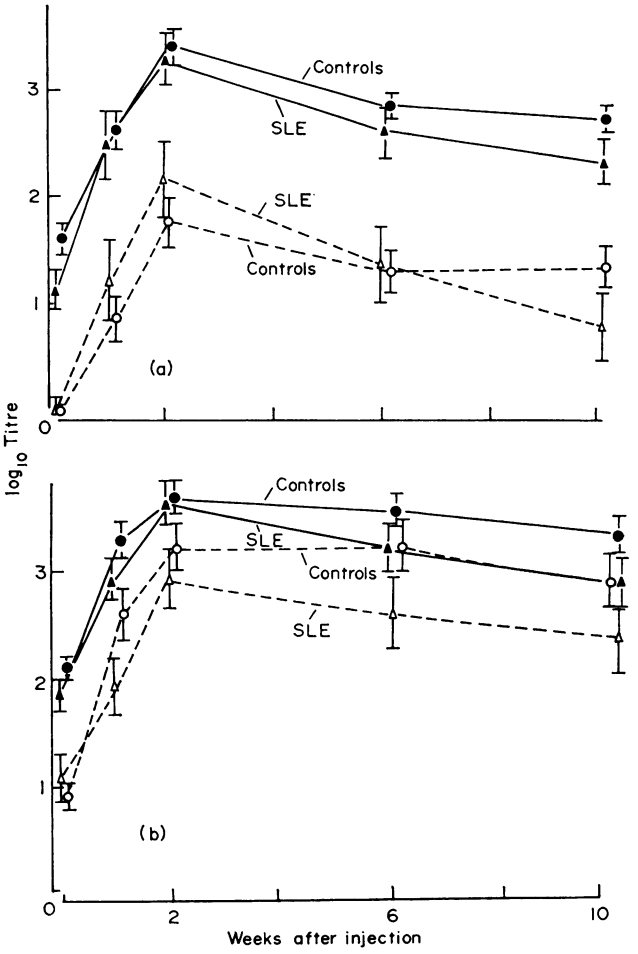

FIG. 1. Geometric mean titres over 10 weeks of total antibody (——) and IgG (ME-resistant) antibody (– – – –) after primary and secondary immunization with flagellin in patients with systemic lupus erythematosus (SLE) (▲) compared with matched 'hospital' controls (●). Vertical bars indicate 1 S.E. of the mean. (a) Primary response; (b) secondary response. There were no significant differences. Codings are the same for all figures.

Lupoid hepatitis. The mean peak titre of total and IgG antibody in the primary response was similar to that of the controls (Fig. 3). The secondary response was not tested.

Allergy. The mean peak titre of total antibody of 4180 was significantly higher ($P<0{\cdot}05$)

than the mean of 1100 for the hospital controls, as was the mean peak titre of IgG antibody, 403 versus 37 ($P<0{\cdot}01$) (Fig. 4). However these allergic patients were in good general health and thus could be more comparable with 'healthy' than 'hospital' controls; when so matched (Fig. 4), there were no significant differences in mean titres either at 2 or 10 weeks after immunization. The secondary response was not tested.

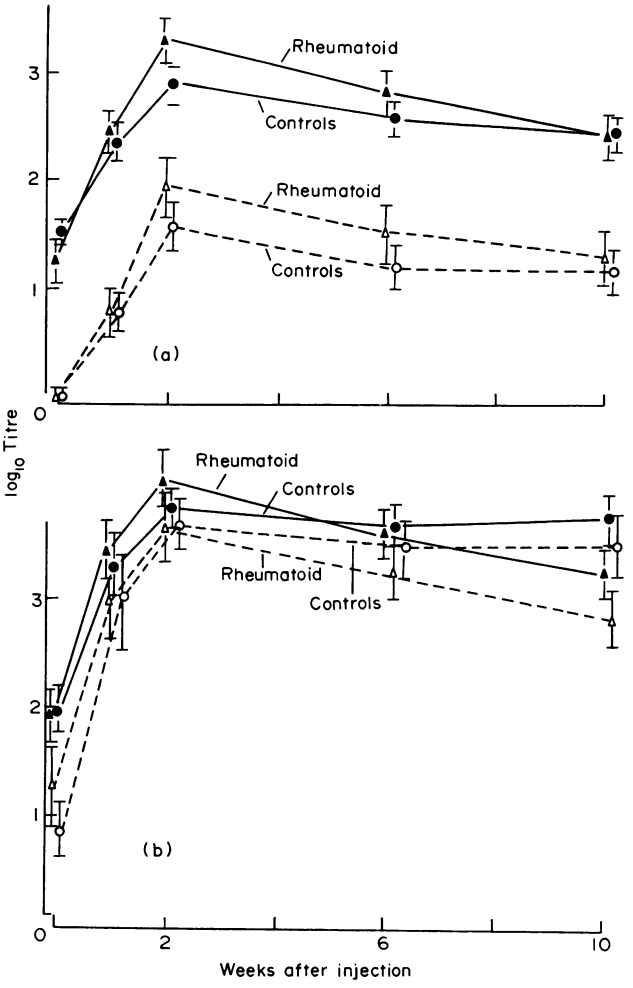

FIG. 2. Geometric mean titres over 10 weeks of total (——) and IgG antibody (– – – –) after primary and secondary immunization with flagellin in patients with rheumatoid arthritis (▲), compared with matched 'hospital' controls (●). (a) Primary response; (b) secondary response. There were no significant differences.

Azathioprine treatment. The mean peak titres of total and IgG antibody of 950 and 38 were lower than those for the controls, 1900 and 85, but these differences would represent only one dilution in titration, and at 10 weeks the mean titres were similar in patients and controls (Fig. 5a). The titres throughout the secondary response were similar in patients and controls (Fig. 5b).

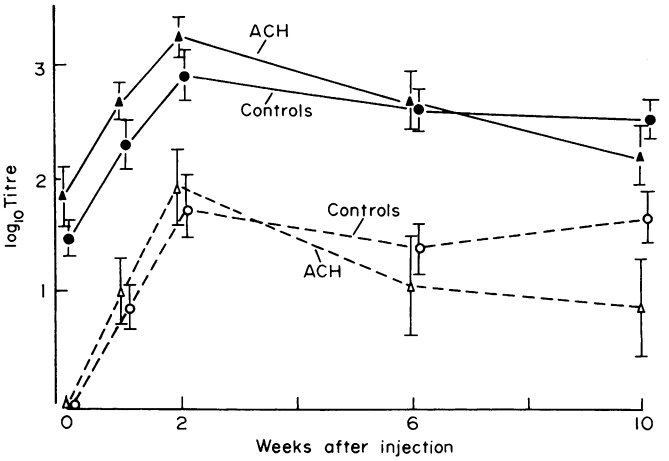

FIG. 3. Geometric mean titres over 10 weeks of total (——) and IgG antibody (- - - - -) after primary immunization with flagellin in patients with active chronic hepatitis (ACH) (▲) compared with matched 'hospital' controls (●). There were no significant differences. Secondary responses were not tested in this group.

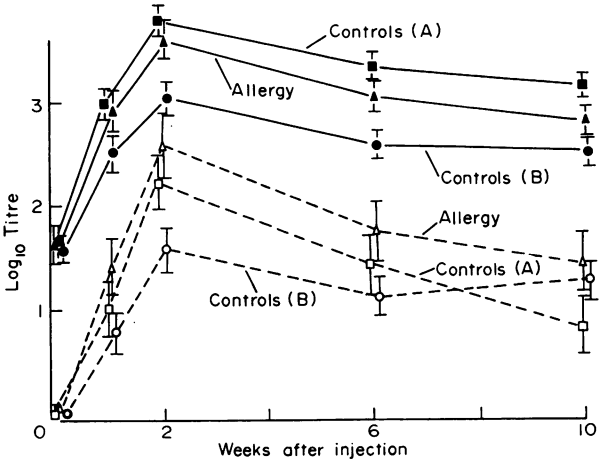

FIG. 4. Geometric mean titres over 10 weeks of total (——) and IgG antibody (- - - - -) in patients with allergy to an extrinsic antigen (▲) after primary immunization with flagellin compared with matched healthy controls (■) and matched 'hospital' controls (●). The response of the allergic patients, although greater than that of hospital controls, did not exceed that of healthy controls. Secondary responses were not tested in this group.

Prednisolone treatment. There were no differences in titres of groups of matched patients receiving and not receiving prednisolone, the mean peak titres in the primary response being 1808 and 1720.

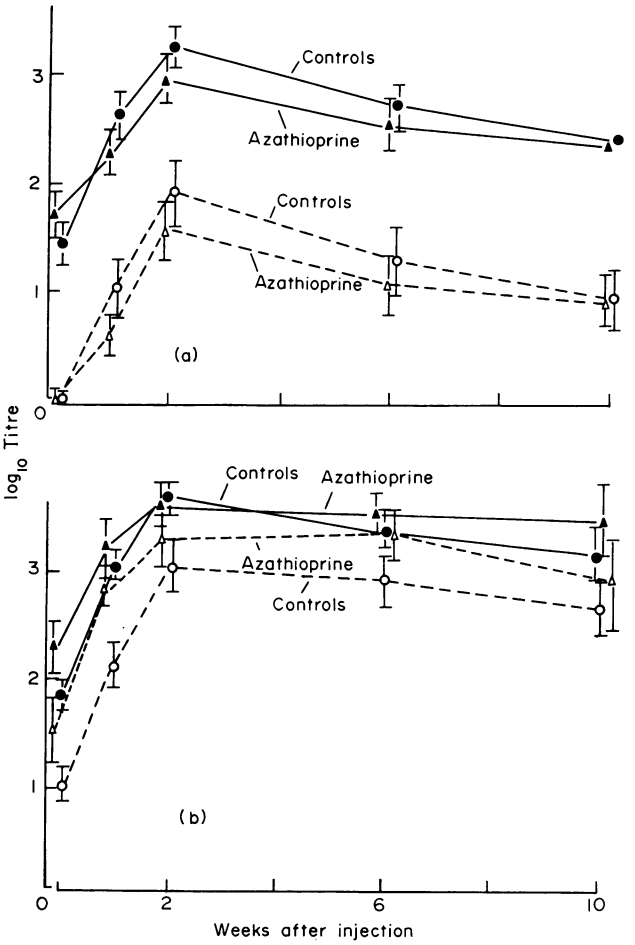

FIG. 5. Geometric mean titres over 10 weeks of total (——) and IgG antibody (– – – –) after primary and secondary immunization with flagellin in patients receiving azathioprine (▲) compared with matched 'hospital' controls (●). (a) Primary response; (b) secondary response. There were no significant differences.

DISCUSSION

Antibody producing capacity, using various 'extrinsic' test antigens, has been studied in several presumed autoimmune diseases but hitherto with inconsistent results.

In SLE a heightened response was reported for brucella vaccine by Meiselas *et al.* (1961), and blood group substances by Zingale *et al.* (1963). However this could not be corroborated for blood group substances and bacterial antigens by Muschel (1961), or for tetanus toxoid by Sarkany (1961) (in cases of discoid lupus) and Barr *et al.* (1964). In a later study Baum &

Ziff (1969) found, comparing patients with SLE with healthy young women, a clearly impaired IgM response to brucella antigen and decreased levels of natural antibody to other bacterial antigens. However in the present study, with controls derived from subjects in hospitals with miscellaneous illnesses rather than healthy subjects, there was no demonstrable difference between the two groups. In other words patients with SLE have the same degree of immunodepression as is found in ill health generally (Rowley & Mackay, 1969).

In rheumatoid arthritis a heightened response was reported for brucella vaccine by Meiselas *et al.* (1961) and for tetanus toxoid by Greenwood & Barr (1960), but normal responses were reported for brucella vaccine by Shearn, Epstein & Engleman (1963) and for tetanus toxoid by Barr *et al.* (1964). A deficient IgM response to sheep erythrocyte stroma and brucella vaccine was reported by Bandilla, Pitts & McDuffie (1970), and 'some suppression' of circulating antibody in patients with severe complicated disease was noted by Denman *et al.* (1970). The responses to brucella vaccine in patients with rheumatoid arthritis were compared with those of their healthy relatives and with age and sex matched controls derived from healthy subjects and relatively well outpatients by Rhodes *et al.* (1969). Antibody-producing capacity was clearly greater in females than males, but there were no significant differences between patients with rheumatoid arthritis, their relatives and the controls. Our findings similarly showed no differences in antibody-producing capacity between patients with rheumatoid arthritis and appropriate controls.

There have been no studies of immune capacity in chronic liver disease apart from the heightened secondary response reported to tetanus toxoid in cirrhosis of the liver by Havens, Myerson & Klatchko (1957). In the present study we found no differences between patients with lupoid hepatitis and hospital controls.

A reasonable consensus, taking in previous observations and our present findings, is that autoimmune disease is not associated with general augmentation of antibody-producing capacity; however it cannot be wholly excluded that such may be present at the onset of illness and thereafter is masked by effects of ill health.

In atopic patients with allergy to spring grass pollen the primary response was significantly greater than that of hospital controls. However, these patients were not ill, and so their response was compared also with that of healthy controls; there were no significant differences. These findings are in line with those of Leskowitz & Lowell (1961) and Salvaggio & Leskowitz (1965) who showed that atopic and normal individuals responded similarly to parenterally administered polysaccharides (dextran and pneumococcal polysaccharide) and protein antigen (purified crystalline bovine ribonuclease), as determined by development of immediate weal and erythema skin reactivity, precipitating antibody and skin-sensitizing antibody.

Our findings in patients treated with azathioprine require consideration. In healthy subjects given azathioprine, Maibach & Epstein (1965) found that the primary response peaked normally but was poorly sustained, and the secondary response at 4 weeks was markedly depressed. In patients with different autoimmune disease treated with azathioprine or amethopterin, Swanson & Schwartz (1967) found various qualitative abnormalities of the primary immune response to haemocyanin, but there seemed no correlation between the degree of immunosuppression and clinical responses to the drug. Previously in this unit Rowley, Mackay & McKenzie (1969) found that allografted patients treated with combined azathioprine and corticosteroids gave a near normal primary response to flagellin, but escondary stimulation with flagellin showed a marked interference with immunological

memory: an early re-challenge at 3 months resulted in no formation of IgG antibody, and late re-challenge after 12 months resulted in a typical primary-type response. However, in the present study on azathioprine-treated patients with autoimmune diseases, there was only slight impairment of the primary response and no interference with memory. The azathioprine dosages for the two groups were similar, but the patients with autoimmune disease were receiving smaller doses of prednisolone and the period of azathioprine treatment before immunization with flagellin was longer.

The absence of demonstrable humoral immunosuppression despite apparent clinical benefit raises the question of the mode of action of azathioprine. Firstly, the patients with autoimmune disease may have had an initially hyperactive response which had been suppressed by treatment with azathioprine to levels comparable with those of the non-azathioprine treated controls, but our data are not in line with this; secondly, the therapeutic effect of azathioprine might depend considerably on an anti-inflammatory action; thirdly, the 'anti-immune' effects of azathioprine may be directed more against cellular than humoral immune responses; fourthly, azathioprine even in relatively low therapeutic doses may be 'tolerogenic' for autoantigens (Schwartz, 1969) without having 'pan-immunosuppressive' effects measurable by responses to an extrinsic antigen; or there may be a combination of the latter three effects.

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