Clin. exp. Immunol. (1982) **49**, 290–296.

Kinetics of specific anti-influenza antibody production by cultured lymphocytes from patients with systemic lupus erythematosus following influenza immunization

D. M. MITCHELL, PENNY FITZHARRIS,‡ R. A. KNIGHT, G. C. SCHILD* & M. L. SNAITH† *I.C.R.F. Human Tumour Immunology Group, School of Medicine, University College London, Faculty of Clinical Sciences, University Street, London; *Division of Viral Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London and †Department of Rheumatology, University College Hospital, Gower Street, London, UK*

(Accepted for publication 19 March 1982)

SUMMARY

Specific antibody responses to influenza viral antigens produced by cultured peripheral blood mononuclear leucocytes stimulated with influenza virus or pokeweed mitogen (PWM) have been measured in seven patients with systemic lupus erythematosus (SLE) before and at time intervals after influenza immunization. Cells from two patients stimulated with influenza virus *in vitro* produced high levels of specific antibody 7 days after immunization. Cells from a third patient produced small amounts of specific antibody at day 14. No antibody was produced by cells from the remaining four patients. Responses were of short duration and were not detectable 1 month after immunization. Specific anti-influenza antibody was induced by PWM only from cells of those patients who responded to virus antigen although absolute levels of antibody produced were not as high. In six patients serum haemagglutination inhibiting antibody to influenza virus was measured, and all six had a greater than four-fold increase. The disparity between *in vitro* antibody production by peripheral blood mononuclear leucocytes and changes in serum antibody suggests that in patients with systemic lupus erythematosus, *in vitro* functions of peripheral blood lymphocytes do not reflect the immune system as a whole.

INTRODUCTION

We have previously reported changes in *in vitro* antibody responses to influenza virus occurring in 11 normal individuals following immunization, who all showed an increase in specific *in vitro* antibody production although there was considerable variation in magnitude and duration (Mitchell *et al.*, 1982). In this study, which was performed concurrently with the study of normal donors, we compared antibody production by virus stimulated or pokeweed mitogen (PWM) stimulated lymphocytes from seven patients with systemic lupus erythematosus (SLE) at various times after immunization with changes in their serum haemagglutinating antibody titres. Virus stimulated antibody production by peripheral blood lymphocytes (PBL) *in vitro* occurred in only three patients, two of whom had a markedly greater response 7 days after immunization than that

‡ Present address: Department of Clinical Immunology, Christchurch Hospital, Christchurch, New Zealand.

Correspondence: D. M. Mitchell, I.C.R.F. Human Tumour Immunology Group, School of Medicine, University College London, Faculty of Clinical Sciences, University Street, London WC1E 6JJ, UK.

0009-9104/82/0800-0290$02.00 © 1982 Blackwell Scientific Publications

shown by PBL from any of the normal individuals studied. PBL from a third patient produced small amounts of specific antibody only on day 14. PBL from the remaining four patients, unlike any immunized normal individual, did not produce antibody. However, the *in vivo* response to influenza immunization in these patients was normal, since in the six patients whose sera was assayed there was a greater than four-fold rise in HI antibody titre following immunization.

MATERIALS AND METHODS

Immunization procedure. Baseline *in vitro* responses to the influenza viruses A/X31 (H3N2) B/HK and PWM were determined, and serum was stored at −20°C for subsequent antibody estimation. Following ethical committee approval, informed consent was obtained from patients, who then received an intramuscular 0·5 ml injection of Merieux Influenza Vaccine (Servier Labs. Ltd.) containing β-propriolactone inactivated *A/Bangkok*/1/79 (H3N2), *A/Brazil*/11/78 (H1N1) and *B/Singapore*/222/79 (Batch U1040-Z). None of the patients experienced significant side effects. *In vitro* responses by PBL to influenza virus and PWM were then determined at intervals.

Lymphocyte culture. In vitro antibody formation by cultured PBL's was determined as described by Callard (1979). Briefly, PBL's were prepared by centrifugation over Ficoll-Hypaque, washed twice in Balanced Salt Solution (BSS), and resuspended in RPMI 1640 containing 25 mM HEPES, 2 g/l bicarbonate and 10% horse serum (GIBCO Biocult, Paisley, Scotland). The cells were cultured at a concentration of 2×10^6/ml in 1 ml volumes in 12 × 75 mm capped tubes (No. 2054 Falcon Oxnard, USA) either alone, or in the presence of optimal concentrations of sucrose density gradient purified A/X31 (A-H3N2) virus, B/HK virus, (kindly provided by Dr J. Skehel, National Institute for Medical Research, Mill Hill) or PWM. (GIBCO, Biocult). The tubes were cultured at 37°C in a 5% CO_2 atmosphere for 6 days, washed twice, and resuspended in 0·5 ml RPMI 1640 containing 20 mM HEPES and 5% fetal calf serum (FCS). After a further 20 hr incubation at 37°C in air, culture supernatants were recovered.

Antibody assay. Specific anti-influenza antibody in culture supernatants was determined by solid phase enzyme immunoassay (EIA), (Callard & Smith, 1981). Briefly, A/X31 influenza virus was absorbed onto microtitre plates (Flow Laboratories), and non-specific binding sites blocked with 1% bovine serum albumin. Culture supernatants were then added to virus coated wells, the plates incubated and then washed extensively. An optimal concentration of goat anti-human IgG coupled to alkaline phosphatase (Voller, Bidwell & Bartlett, 1976) was then added, and following further incubation and washing, nitrophenol phosphate substrate (Sigma) was added to each well. The optical density (OD) of each well at 405 mm was determined on a Titertek Multiskan (Flow Laboratories). Results from triplicate cultures were expressed as ng/ml of specific antibody calculated from a standard curve of logit transformed OD_{405} versus ln (concentration) of specific anti-A/X31 antibody. Values below 2 ng/ml were not considered to be significant.

Serum antibody levels. Haemagglutination inhibition (HI) tests were carried out by standard microtitre methods (WHO, 1959).

RESULTS

In vitro antibody responses to A/X31 and B/HK influenza viruses by PBL from seven patients with SLE were measured before and at time intervals after influenza immunization (Fig. 1). At day seven PBL from two patients produced concentrations of specific antibody greater than those seen from PBL in any of 11 normal individuals studied concurrently (Mitchell *et al.*, 1982). By day 14 these two high responses had more than halved and had returned to almost zero by day 28. Of the remaining five patients, only one had a low level of response and this was at day 14 only. No antibody was produced by PBL from the three patients in whom responses were measured at day 56. PBL from the remaining four patients produced no antibody at any time during the study.

Specific anti-A/X31 antibody production following polyclonal activation of PBL with PWM was also determined (Fig. 2). A similar profile of response was observed in the two patients with

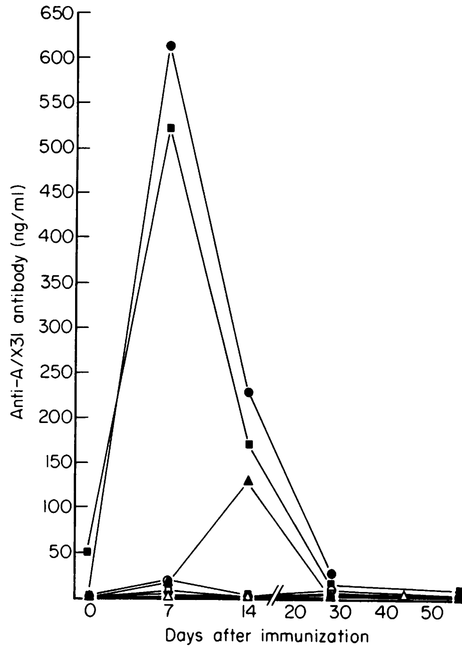

Fig. 1. Kinetics of specific anti-A/X31 antibody production by PBL stimulated with A/X31 virus from seven SLE patients following influenza immunization. Antibody production by cells from individual patients is shown using the same symbol for each patient in all figures.

high responses following A/X31 stimulation, although the levels achieved with mitŏgen stimulation were not as high on day 7 as with virus stimulation. No response was seen to PWM among the five patients who also failed to respond A/X31 stimulation.

Total immunoglobulin levels in supernatants from cultures stimulated with A/X31 were very low, being of the same order as specific antibody produced.

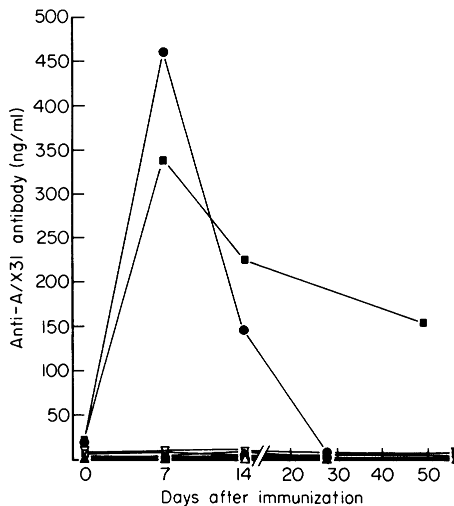

Fig. 2. Specific anti-A/X31 antibody production by PBL stimulated with PWM from seven SLE patients following influenza immunization.

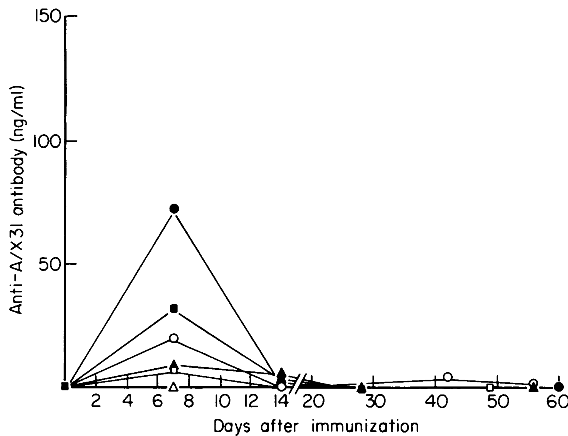

Fig. 3. Specific anti-A/X31 antibody production by PBL in the absence of specific antigenic or mitogenic stimuli.

In control tubes to which neither virus nor PWM had been added, spontaneous specific antibody production was observed at day 7 by cells from the two patients who showed high virus stimulated antibody production. Lower spontaneous antibody production was also seen in three of the non-responders (Fig. 3).

Specific anti-B/HK antibody was measured in supernatants of PBLs from all patients stimulated *in vitro* with B/HK virus and PWM, as well as supernatants from unstimulated control cultures (Fig. 4). Results paralleled those obtained with A/X31 virus in that the two patients with high responses to A/X31 also responded to B/HK, although absolute levels of antibody production were not as great.

Serum haemagglutination inhibiting (HI) antibody titres were determined to *A/Bangkok*/1/79 (H3N2) and *B/Singapore*/222/79 in six of the patients (Table 2). A greater than four-fold increase in titre occurred to the A type virus in all except patient C who had a high pre-immunization titre

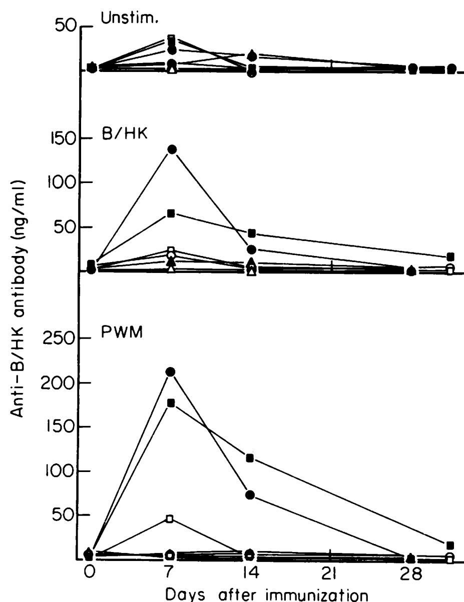

Fig. 4. Kinetics of specific anti-B/HK antibody production by PBL either unstimulated, stimulated with B/HK virus, or PWM.

Table 1. Clinical state, laboratory investigations and treatment of patients with SLE

	Patients						
	A	B*	C	D	E	F*	G
ARA criteria†	3 5 7 11 12	7 8 12 14	2 4 7 12 14	4 5 8 10 12 13 14	7 8 13 14	7 8 10 12	7 8 12 14
Clinical state‡	2	2	3	2	2	2	2
Visual analog§	4	6	8	2	0	2	7
ESR (< 10 mm/hr)	26	12	119	86	68	25	33
DNA binding (0–25 units/ml)	37	278	970	114	800	92	112
CIC (< 100 μg/ml IgG)	278	105	239	854	261	193	206
C3 (840–1652 mg/l)	857	2071	263	632	1047	1181	1069
Lymphocyte count (above $1{\cdot}5\times10^9$/ml)	1·2	0·9	0·6	1·1	0·6	1·3	0·7
Drug treatment							
Prednisone (mg per day)	10 mg on alt. days	10–20	10–20	15–30	0–25	10–20	10–20
Duration	18 mths	4 yr	3 yr	3 yr	2 yr	1 yr	2 yr
Azathioprine dose prior to trial (mg/day)	100	—	150	—	125	125	100–125
Duration	1 yr					0·5 yr	2 yr
I.V. methyl prednisolone bolus (1 g on 3 successive days)	July Jan	—	Sept 80	Feb 79 Feb 80 July 80	June 80	Sept 80 Dec 80	March 80
Cyclosporin	Oct 79	Dec 79	—	—	—	—	—
Chloroquin	—	Intermittent 1960–1977	—	—	—	—	—

* Patients B, and F had high *in vitro* antibody responses to influenza virus.

† ARA criteria present (Cohen *et al.*, 1971): (1) Facial erythema; (2) Discoid lupus; (3) Raynaud's phenomenon; (4) Alopecia; (5) Photosensitivity; (6) Oral or nasal ulcers; (7) Arthritis without deformity; (8) Profuse proteinuria; (9) Cellular casts; (12) Pleuritis/pericarditis/abnormal respiratory function tests; (13) Psychosis/convulsions; (14) Cytopenia.

‡ Clinical state (1) = inactive; (2) = one or two present of; (3) = three or more present of; (pleurisy; pericarditis; arthritis; glomerulonephritis; vascular skin disease; cerebral involvement; myositis).

§ Visual analog scale. At the beginning of the study patients were asked to mark how they felt on a 10 cm line labelled 'very good' at one end, 'very bad' at the other. (Very good = 0; very bad = 10).

of 160. Of the two patients who had high *in vitro* responses, one also showed the highest increase in serum antibody. The serum changes in antibody titre were similar (range 1·5–128-fold increase) to changes seen in the group of 11 normal individuals studied concurrently (range 0–48-fold increase).

Before the study, clinical details were obtained at interview from the seven patients (Table 1). All had at least four ARA criteria at some time in the past, and had active disease at the time of the study. Laboratory tests showed that all but one had an elevated sedimentation rate, and circulating immune complexes. DNA binding (Farr technique) was elevated in all patients and two had diminished levels of complement C3. All had varying degrees of lymphopenia. All patients were taking oral prednisone in doses ranging from 10 mg alternate days to 30 mg/day. Five patients had

Table 2. Serum haemagglutination inhibiting (HI) antibody titres to A/BK/1/79 H_3N_2 and B/Sing/222/79

Patients		Days after immunization 0	7	14	28	Increase
A	B/Sing	10	60	480	320	×48
	A/BK	10	10	240	240	×24
B*	B/Sing	10	—	480	320	×48
	A/BK	30	—	640	120	×21
C	B/Sing	10	—	10	480	×48
	A/BK	160	—	240	80	×1·5
D	B/Sing	15	40	640	480	×42
	A/BK	15	20	480	320	×32
E	B/Sing	10	10	10	10	×0
	A/BK	40	40	480	10	×12
F*	B/Sing	10	—	480	480	×48
	A/BK	20	160	2560	5120	×128

* Individuals with high *in vitro* responses.

recently been taking azathioprine but this was stopped 2 weeks before and during the study period. Six of the patients had received intravenous methyl prednisolone, two cyclosporin, and one chloroquin, at some time in the past. There was no clear difference in clinical history, laboratory investigations or drug treatment between the two patients with high *in vitro* responses and those with no responses.

DISCUSSION

In this study the kinetics of specific antibody production by virus and mitogen stimulated PBLs from seven patients with active SLE have been compared with changes in serum HI antibody, following influenza immunization. Only three patients' PBL responded *in vitro*, and of these, two had responses at day 7 which were higher than any responses seen in a group of 11 normal individuals studied concurrently (Mitchell *et al.*, 1982). No antibody was produced by cells stimulated *in vitro* from the remaining four patients. All the patients however showed significant rises in HI titres to both A type or B type virus. One of the two patients who produced high levels of specific antibody *in vitro* showed the highest rise in serum HI titre seen in the combined group of SLE patients and normals.

Although the patient numbers are small, two abnormal patterns of response were seen—non-responsiveness and short lived hyper-responsiveness.

Lack of response by PBLs from four patients stimulated with virus or mitogen *in vitro* is not consistent with the increases in serum HI titre. In the group of immunized normal donors, increase in serum HI titres was invariably associated with increase in *in vitro* response. This suggests that *in vitro* studies of the lymphocyte pool circulating in peripheral blood are not representative of the immune system as a whole, in these patients.

Apparent lack of response by PBLs *in vitro* may also be secondary to changes in the T/B cell ratios which have been reported in some lupus patients (Raveche & Steinberg, 1979).

Cells from two patients in the present study produced abnormally high amounts of antibody when stimulated with A/X31 *in vitro*. This was not due to carry over of serum antibody as cells were extensively washed at the beginning and end of the culture and, the high levels of *in vitro* antibody were not sustained, whereas serum levels were. Supranormal antibody responses in SLE patients have been reported to blood group substances (Zingale *et al.*, 1963) and penicillin (Harris & Vaughan, 1960). Other studies, however, have found normal antibody responses to tetanus toxoid

(Sarkany, 1961), Brucella (Abe & Homma, 1971), Rickettsia (Lee *et al.*, 1960), blood group antigen and anti-streptolysin O (Muschel, 1961), and other reports claim low response to Brucella (Baum & Ziff, 1969). These two abnormal patterns of response seen in SLE suggest that in some patients antigen specific lymphocytes capable of generating a secondary antibody response to exogenous antigens appear briefly in peripheral blood following immunization, whereas in others, cells that can react to such antigens after immunization do not appear in the circulation but are confined to lymphoid tissue.

Changes in serum haemagglutinating antibody after influenza immunization in SLE patients have been studied (Brodman *et al.*, 1978; Louie *et al.*, 1978; Ristow, Douglas & Condemi, 1978; Williams *et al.*, 1978). Broadly, all report rises in patients' serum HI titre comparable with those of normal donors. In this study, the titres of HI antibody against either A/X31 or B/HK viruses, or both, showed increases similar to those of normal donors.

No clinical distinction can readily be made between the two patients who had high *in vitro* responses, and the four non-responders (Table 1), although the two hyper-responders had the two lowest ESR and CIC values of the seven patients in the study. All patients had been taking similar doses of oral steroids for at least a year, and one hyper- and four non-responding patients had received azathioprine. All patients were classified clinically as having moderately active disease, except one who had severe disease. This patient did not produce *in vitro* antibody.

This study shows that, despite normal rises in serum HI titres *in vivo*, cells from a small, but clinically similar group of lupus patients show two different pathological responses to virus antigen *in vitro* after influenza immunization. Although any general conclusion must be qualified by reservations about the small number of patients it was possible to study, the data are consistent with considerable variations in lymphocyte function between individual patients, which may be of pathogenetic importance in this disease.

REFERENCES

Abe, T. & Homma, M. (1971) Immunological reactivity in patients with systemic lupus erythematosus. Humoral antibody and cellular immune responses. *Acta Rheumatol. Scand.* **17,** 35.

Baum, J. & Ziff, M. (1969) Decreased 19S antibody response to bacterial antigens in systemic lupus erythematosus. *J. clin. Invest.* **48,** 758.

Brodman, R., Gilfillan, R., Glass, D. & Schur, P.H. (1978) Influenza vaccine response in systemic lupus erythematosus. *Ann. Int. Med.* **88,** 735.

Callard, R.E. (1979) Specific *in vitro* antibody response to influenza virus by human blood lymphocytes. *Nature,* **282,** 734.

Callard, R.E. & Smith, C.M. (1981) Histocompatibility requirements for T cell help in specific *in vitro* responses to influenza virus by human blood lymphocytes. *Eur. J. Immunol.* **11,** 20.

Cohen, A.S., Reynolds, W.E., Franklin, F.C., Kulka, J.P., Ropes, M.W., Shulman, L.E. & Wallace, S.L. (1971) Preliminary criteria for the classification of systemic lupus erythematosus. *Bull. Rheum. Dis.* **21,** 643.

Harris, J. & Vaughan, J. (1960) Penicillin antibody in disease. *J. clin. Invest.* **39,** 995.

Lee, S.L., Mieselas, L.E., Zingale, S.B. & Richman, R. (1960) Antibody production in systemic lupus erythematosus and rheumatoid arthritis. *J. clin. Invest.* **39,** 1005.

Louie, J.S., Nies, K.M., Shoji, K.T., Fraback, R.C., Abriss, C., Border, W., Cheng, J.D. & Imagawa, D. (1978) Clinical and antibody responses after influenza immunisation in systemic lupus erythematosus. *Ann. Int. Med.* **88,** 790.

Mitchell, D.M., Fitzharris, P., Knight, R.A. & Schild, G.C. (1982) Kinetics of specific *in vitro* antibody production following influenza immunization. *Clin. exp. Immunol.* **48,** 491.

Muschel, L.H. (1961) Systemic lupus erythematosus and normal antibodies. *Proc. Soc. exp. Biol. Med.* **106,** 622.

Raveche, E.S. & Steinberg, A.D. (1979) Lymphocytes and lymphocyte functions in systemic lupus erythematosus. *Sem. Haematol.* **16,** 344.

Ristow, S.C., Douglas, R.G. & Condemi, J.J. (1978) Influenza vaccination of patients with systemic lupus erythematosus. *Ann. Int. Med.* **88,** 786.

Sarkany, I. (1961) Circulating antibody formation in lupus erythematosus. *Arch. Dermatol.* **84,** 372.

Voller, A., Bidwell, D.E. & Bartlett, A. (1976) Enzyme immunoassays in diagnostic medicine. Theory and Practice. *Bull. WHO,* **53,** 55.

Williams, E.W., Steinberg, A.D., Reinertsen, J.L., Klassen, L.W., Decker, J.L. & Dolin, R. (1978) Influenza immunisation in systemic lupus erythematosus. A double blind trial. *Ann. Int. Med.* **88,** 729.

World Health Organization Expert Committee on Respiratory Virus Diseases (1959) *WHO Tech. Rep. Ser.* 170.

Zingale, S.B., Sanchez Avalos, J.C., Andrada, J.A., Stringa, S.G. & Manni, J.A. (1963) Appearance of anticoagulant factors and certain autoimmune antibodies following antigenic stimulation with blood group substances in patients with systemic lupus erythematosus. *Arthrit. Rheum.* **6,** 581.