Correlation of disease activity and drug therapy with the autologous mixed lymphocyte reaction in rheumatoid arthritis

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SUMMARY The response of peripheral blood lymphocytes (PBL) to autologous synovial fluid lymphocytes (SFL) from patients with rheumatoid arthritis and Reiter's syndrome was investigated in an autologous mixed lymphocyte reaction (AMLR). SFL were found to be poor responders but strong stimulators of autologous and allogeneic PBL compared with autologous PBL. The plastic-adherent (macrophage) cells from the SFL were found to be highly stimulatory to autologous PBL, particularly when the adherent cells were removed from the responding PBL. The stimulation of these PBL non-adherent cells by SFL adherent cells follows two main trends: either no stimulation, or higher stimulation than using unseparated SFL and PBL. Patients in the high stimulator group were taking non-steroidal anti-inflammatory drugs while those in the low responder group were taking, in addition, second-line drugs such as D-penicillamine or gold. Autologous serum was found to inhibit the AMLR and this is probably due to drug metabolites in patients' sera. Initial results show that the AMLR in individual patients is highly correlated, over time, with the erythrocyte sedimentation rate (ESR).

Key word: synovial fluid.

The autologous mixed lymphocyte reaction (AMLR) is an in-vitro assay which measures the response of T cells to non-T cell surface membrane antigens, coded for by the HLA-D region. The significance of the AMLR in vivo in normal individuals is uncertain, but there is increasing evidence from in-vitro studies that it plays an important part in the regulation of autoimmune events.

The AMLR demonstrates two fundamental features of immune processes; memory and specificity.¹ It generates cytotoxic lymphocytes¹ and perhaps natural killer cells,² and produces suppressor cells^{3,4} capable of regulating the production of immunoglobulin by B cells^{5,6} and the response of T cells to antigen and mitogen.⁷ If the AMLR is a homeostatic regulator of the immune system, then it is possible that any increase in autoreactivity, as in autoimmune disease, may be due to a breakdown of

immunoregulation and altered AMLR. This study suggests such a relationship.

An impairment of the AMLR has been demonstrated in autoimmune disorders such as systemic lupus erythematosus, primary biliary cirrhosis, and Sjögren's syndrome,⁸ and in polymyositis/dermatomyositis patients,⁹ conditions in which both cellular and humoral immunity are altered. Disturbances in immunoregulation have also been detected in rheumatoid arthritis (RA) and are believed to be due to a lack of suppressor T cell activity⁸ or T cell regression activity.¹⁰ An increased ratio of helper/suppressor T cells with a concomitant presence of strongly DR⁺ macrophage-like cells,¹¹ which allow autostimulation to persist, have also been described in RA patients. Thus the AMLR may be a useful in-vitro model for the investigation of cellular interactions important in immunoregulation in patients with RA or other forms of arthritis, such as Reiter's syndrome.

In this study the AMLR has been used to assay the proliferation of peripheral blood lymphocytes

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(PBL) in response to stimulation by autologous synovial fluid lymphocytes (SFL). Previous studies applying the AMLR of PBL to autologous SFL in RA patients have been equivocal about whether the AMLR is stimulated^{8 12 13} or suppressed¹⁴ and have shown either lack of correlation¹³ or correlation^{12 15} with disease activity. These conflicting results may have been due in part to the use of techniques known to affect cell function. For example, fetal calf serum¹⁶ and sheep red blood cells (SRBC) used to fractionate cells are stimulatory to T cells,¹⁷ and the use of hyaluronidase to pretreat synovial fluid samples has previously been found to decrease the response of lymphocytes to mitogens.¹⁸ By using unfractionated lymphocytes and reducing cell handling an attempt has been made to minimise conditions which may inadvertently alter the immune response under study.

Materials and methods

PATIENTS

Twenty-one patients with classical or definite RA (12 females, nine males) and three with Reiter's syndrome were studied. Most patients were receiving non-steroidal anti-inflammatory drugs (NSAID) and some were on second-line drugs as well. Patients on 3 mg of prednisone or more were excluded from the study. Synovial fluid and peripheral blood samples were collected at the time of aspiration and lymphocytes were separated immediately. Two healthy donors acted as a source of control lymphocytes (C1 and C2) for each patient's AMLR.

Isolation and growth of lymphocytes were carried out in culture medium RPMI 1640. This medium was supplemented with 5% pooled human AB serum during isolation procedures, but to maintain growth either 20% pooled AB or autologous serum was used. Lymphocytes from synovial fluid and peripheral blood were isolated by centrifugation on a Ficoll-Hypaque gradient and the cells were not usually fractionated any further before culture. All cells were adjusted to $1.5 \times 10^9/l$ prior to plating into microtitre trays. Where necessary, T and B cells were separated from the mononuclear cells by standard rosetting techniques.¹⁹ The plastic-adherent cells (predominantly macrophages) were isolated from the SFL and PBL populations by the method of Seitz *et al.*²⁰ and identified by non-specific esterase stain. Primary one-way autologous (AMLR) and allogeneic (aMLR) mixed lymphocyte reactions were performed in 96-well round-bottomed microtitre plates (Disposable Products, Brisbane, Australia) in a checkerboard design with $1.0\text{--}1.5 \times 10^5$ responder cells and an equal number of mitomycin-C treated

(40 mg/l cells for 20 minutes at 37°C) stimulator cells in a final volume of 200 μl /well. All cultures were grown at 37°C, 5% CO₂ in air for six days. Phytohaemagglutinin (PHA-P, Difco, Detroit, Michigan) was added to each responder cell (10 mg/l) alone, 72 hours prior to harvest. Proliferative responses were measured by adding 1 μCi /well of ³H-thymidine (New England Nuclear, Boston, Mass.) for the final 18–20 hours of culture. The cells were harvested with a Skatron multisample harvester, and the incorporation of ³H-thymidine determined by standard liquid scintillation counting techniques. Results are expressed as mean disintegrations per minute (dpm) in

Fig. 1 Incorporation of ³H-thymidine into SFL, PBL, PHA-treated SFL and PBL of RA (●) and Reiter's disease patients (▽). Shown are the mean and SEM. The mean incorporation into SFL was 11 000 dpm; PBL, 2228 dpm, SFL + PHA, 20 500 dpm, and PBL + PHA, 64 000 dpm, were significantly different ($p < 0.01$). Each point represents the mean of quadruplicate cultures.

quadruplicate cultures minus the background dpm in responder cells when cultured alone. All comparisons were analysed by either a paired or an unpaired Student's *t* test.

Results

Cultures revealed that the optimal ratio of respon-

Fig. 2 Allogeneic stimulation and response of SFL and PBL from RA patients. Results are shown as the mean $\pm$ SEM of ^3H -thymidine incorporation. Stimulation of control cells (C) by mitomycin-C treated SFL (mSFL) gave 200 100 dpm, significantly higher than mitomycin-C treated PBL (mPBL/C) which gave 116 624 dpm in a paired *t* test ($p < 0.001$). The response of SFL (mC/SFL) was 93 500 dpm, significantly less than mC/PBL which gave 122 572 dpm ($p < 0.005$). Each point is the mean of quadruplicate cultures.

der to stimulator cells was 1:1 and that the optimal response occurred on day 6 in both the AMLR and aMLR.

SFL were found to be good stimulators in the AMLR but poor responders to PHA and allogeneic or autologous stimulation. The following results confirmed that the SFL were already activated

Fig. 3 The AMLR (mSFL/PBL) of RA (●) and Reiter's disease patients (▽) in pooled AB, and autologous serum is compared with the other autologous combination mPBL/PBL. RA and Reiter's disease patients who were not taking medication or who had medication withdrawn 48 hours prior to assay, are represented by open symbols (○, ▽). Shown are the mean (dpm) $\pm$ SEM, and each point is the mean of quadruplicate cultures. The mean incorporation for the AMLR of RA patients is 30 000 dpm (Reiter's disease patients 43 500 dpm) in pooled AB serum, but only 3700 dpm in the autologous serum of patients on drugs ($p < 0.05$). The AMLR is significantly larger ($p < 0.01$) than mPBL/PBL (1500 dpm).

(1) the increased incorporation of ^3H -thymidine into resting SFL compared with autologous PBL was some three times greater on average (Fig. 1); (2) PHA stimulation of SFL was significantly lower compared with autologous PBL ($p<0.01$); (3) SFL as stimulators in the aMLR induced a significantly larger response than autologous PBL ($p<0.001$) (Fig. 2); and (4) SFL responded less than autologous PBL ($p<0.005$) to allogeneic cells (Fig. 2).

In pooled AB serum (Fig. 3) the only cell combination that showed significant autologous stimulation was with mitomycin-C treated SFL as stimulator and PBL as responder (mSFL/PBL). The mean response of this AMLR was 30 000 dpm for patients with RA and 43 500 dpm for patients with Reiter's disease. This response was statistically larger than that for other autologous combinations such as mPBL/PBL, mSFL/SFLm and mC/C (RA, $p<0.01$; Reiter's disease, $p<0.001$). The patients with Reiter's disease showed less variation in their AMLR response than RA patients.

Cultures with sonicated SFL revealed negligible stimulation (mean incorporation 900 dpm) compared with the mean AMLR (30 000 dpm).

In nine out of 12 RA patients the addition of autologous serum severely decreased the AMLR to control levels, compared with pooled AB serum ($p<0.05$). This autologous serum was also found to suppress the response of lymphocytes to PHA stimulation. However, as shown in Fig. 3, three patients with RA and three with Reiter's disease who had not taken any medication or who had ceased

medication at least 48 h prior to assay had an AMLR which was relatively unchanged by incubation in autologous serum.

Removal of the adherent cells from the stimulating population (mSFL) in the AMLR caused a significant decrease in thymidine incorporation in eight of the 12 cultures of RA patients but was not significantly affected in four of the RA patients (Table 1). In these four cultures stimulation in the AMLR resided in the non-adherent fraction of SFL. The mean incorporation of these mSFL non-adherent/PBL mixtures was 14 829 dpm, about half of the unfractionated mSFL/PBL combination (27 706 dpm). Adherent cells removed from the responder PBL population when stimulated by unfractionated mSFL cells in the AMLR resulted in either a decreased, increased, or indifferent response (Table 1). In two of the 11 cultures from patients with RA there was a significant decrease in the AMLR response, while in six patients removal of the macrophages from the responding PBL population had no effect; but in three cultures there was a greatly increased response, far larger than the initial AMLR. The stimulation of PBL by PBL (adherent) cells was negligible (Table 2). In seven of the 10 RA patients the mean stimulation of PBL by autologous SFL (adherent) was 10 390 dpm, 10 times larger than PBL (adherent) cells, but significantly less than with unfractionated mSFL. In three cases the mSFL (adherent) cells were not significantly different, compared with mSFL, in their stimulating capacity (Table 2).

Table 1 Effects of removal of the plastic-adherent cells on the AMLR

Patient	Sex	Drugs	ESR (mm/h)	mSFL/PBL	mSFL (non-adherent) /PBL	p	mSFL/PBL (non-adherent)	p
1	M	Gold and NSAID	66	11 023±1088	3 489±1204	<0.05		
2	M	Gold	51	8 170±2044	1 814±126	NS	253±152	<0.01
		Pencill.	80	19 434±2173	7 120±1080	<0.01	31 919±5923	NS
3	F	NSAID	80	36 607±6423	1 358±1140	<0.05	8 396±2428	NS
4	M	NSAID	42	68 121±1098	60 685±3421	NS	66 195±3756	NS
5	M	Penicill. and NSAID	47	16 384±2668	4 792±131	<0.05	15 629±7761	NS
6	F	NSAID	46	31 322±3924	15 710±2203	<0.01	32 818±5146	NS
7	F	Pencill. and NSAID	48	50 738±3794	20 014±1955	<0.005	22 668±62	<0.005
		and NSAID	20	2 234±326	66±98	<0.01	15 536±1001	<0.001
8	M	Penicill. and NSAID	93	9 264±2578	6 271±306	NS	19 043±113	<0.05
9	M	NSAID	25	16 616±1783	7 036±588	<0.05	37 098±1525	<0.01
10	M	NSAID	60	62 558±2299	49 594±4607	NS	47 089±9781	NS
Mean±SEM				27 706±6419	14 829±5727		26 968±5644	

Results are the mean (dpm) of quadruplicate cultures, and results of the non-adherent cells are compared with each patient's AMLR using a Student's paired t test.

NS=Not significant.

Penicill.=Penicillamine. NSAID=Non-steroidal anti-inflammatory drugs. Patients 10 in Tables 1 and 2 are different subjects.

Table 2 The effect of plastic-adherent cells in the AMLR

Patient	Sex	Drug	ESR (mm/h)	mSFL/PBL	mSFL (adherent) /PBL	p	mSFL (adherent) /PBL (non-adherent) adherent	p	mPBL (adherent) /PBL
1	M	Gold and NSAID	66	11 023±1088	1 842±336	<0.001	1 459±1176	<0.001	
2	M	Penicill.	80	19 434±2173	3 220±701	<0.01	61 151±16783	<0.05	204±102
3	F	NSAID	80	36 607±6423	13 862±4652	NS	32 653±1098	NS	
4	M	NSAID	42	68 121±1098	52 323±1350	NS	50 001±2326	NS	1435±1176
5	M	Penicill. and NSAID	47	16 384±2668	1 128±131	<0.005	2 270±392	<0.001	812±828
6	F	NSAID	46	31 322±3924	21 320±2381	NS	55 678±2400	<0.005	850±392
7	F	Penicill.	48	50 738±3794	56±477	<0.001	3 208±1051	<0.001	0
8	M	Penicill.	93	9 264±2578	2 243±180	<0.05	1 455±180	<0.001	0
9	M	NSAID	25	16 616±1783	2 584±484	<0.005	4 767±1795	<0.05	
10	F	NSAID	42	27 631±921	5 327±2346	<0.01	58 179±1325	<0.001	
Mean±SEM				28 714±5948	10 390±5121		27 082±8497		825±251

Results are the mean (dpm) of quadruplicate cultures, and the adherent cell stimulation for each patient is statistically compared with their AMLR (mSFL/PBL). NS=Not significant. Pencill.=Penicillamine. NSAID=Non-steroidal anti-inflammatory drugs.

Fig. 4 ³H-Thymidine incorporation in the AMLR (mSFL/PBL) and stimulation by the plastic-adherent cells of the SFL, mSFL(adh), in the AMLR. Shown is the mean (dpm)±SEM and all points are the mean of quadruplicate cultures. The mSFL(adh) are incubated with PBL (non-adh) cells. The mean AMLR in these RA patients is 27 700 dpm. The mSFL(adh)/PBL(non-adh) response is divided into high responders (58 300 dpm) and low responders (2600 dpm) which recently showed an increased or decreased ESR (of > 20 mm/h) respectively.

Fig. 5 Correlation of the AMLR with ESR in RA patient 7 (●), $r=0.98$, $p<0.05$ and patient 2 (■), $r=0.97$, $p<0.05$ on three separate occasions.

cases (RA) the AMLR was markedly increased (mean 58 300 dpm).

The patients were now observed to form two distinct groups where the AMLR was altered with removal of PBL macrophages. Those patients in the upper response group all had very active disease and a recent increase in their ESR (greater than 20 mm/h). All three patients were taking NSAID and one patient took penicillamine. The lower group of patients were on gold or penicillamine, and only one patient out of five was taking NSAID alone. Their low AMLR correlated with a recent decrease in ESR.

Though the overall data showed no correlation of the AMLR with ESR, serum rheumatoid factor, or synovial fluid C-reactive protein at the time of assay, in two cases sequential studies revealed a significant correlation of ESR with the AMLR (Fig. 5).

Discussion

In keeping with other studies employing surface markers and immunohistochemical stains of lymphocyte activation^{21 22} in RA the SFL in the present study were found to be significantly more activated than autologous PBL in both RA and Reiter's disease patients. The origin of the activated SFL in RA is uncertain, though it has been suggested that they arise from the superficial layer of synovium.²³

In the AMLR the SFL were potent stimulators of

autologous PBL. The reason for this may be that the SFL and PBL express some class II region disparity due to altered or previously unexpressed antigens. It has been found that for the production of immunoglobulin-producing cells (via induction of helper T cells), class II region disparity is essential in the primary MLR.²⁴ The cause of this disparity may be explained by the modified-self theory,²⁵ in which autostimulation can occur by the presentation of modified-self antigens of cross-reacting microbial antigens with class II products, by antigen presenting cells. The very low levels of stimulation by sonicated SFL in the AMLR suggest that an active cell product is required, either soluble or membrane bound. The addition of IL-2 to activated lymphocytes is known to lead to clonal expansion,²⁶ though IL-2 production is depressed in RA.²⁷

Studies on tissue sections^{11 28} suggest that the presence of strongly HLA-DR⁺ macrophage-like cells are the cause of stimulation of autologous synovial T cells and these are supported by studies of RA SFL (adherent) in this report. Removal of the SFL (adherent) cells greatly reduced the AMLR in RA patients, indicating that these synovial macrophages were stimulatory. The SFL (non-adherent) cells were also stimulatory but not to the same extent as unseparated SFL. The SFL (adherent) cells alone were also capable of inducing significant stimulation when incubated with autologous PBL. When the mean value for the SFL (adherent) cell stimulation was added to that of SFL (non-adherent) cells, the combined stimulation was slightly less than that of unfractionated SFL. This implies a synergistic relationship between synovial fluid lymphocyte and macrophage cells. This communication between cell types involved in immunoregulatory responses would be expected to occur in the AMLR, creating either an increased or decreased response above that of either cell type alone. A previous study found that macrophages are capable of either stimulation or suppression of immune responses.²⁹ This is confirmed in part in the current study, which suggests that SFL (adherent) cells are stimulatory, while PBL (adherent) cells are not and in fact may be inhibitory in the AMLR. Removal of adherent cells from the PBL population greatly increased the response of some RA patients' PBL to either unfractionated SFL (Table 1) or SFL (adherent) cells. The incubation of mSFL (adherent) with PBL non-adherent cells resulted in some RA patients in an AMLR much larger than the combination mSFL/PBL. This implies that the PBL (adherent) cells recognise the SFL as foreign and are either producing an inhibitory factor, such as prostaglandin E₂,^{20 30 31} or are inducing suppressor T cell activity in the PBL population.

The division of the response of non-adherent PBL to SFL (adherent) cells into high and low responders may be due to drug therapy. Second-line drugs such as gold, D-penicillamine, and steroids have been shown to affect the AMLR and are proposed to affect the antigen-presenting function of macrophages by binding to HLA-DR determinants.^{32 33} These drugs are therefore potentially capable of decreasing the AMLR. This response to mSFL (adherent) cells also seems to be correlated with relative (recent) changes in ESR.

The findings of a possible correlation between the AMLR and the relative changes in the ESR may explain the lack of correlation between absolute values of the ESR and the AMLR. The AMLR was found to be highly correlated with ESR in two patients with RA, over time, and hence may be a useful predictor of subsequent disease activity, though a prospective study would be required to confirm this. ESR and other laboratory indices such as C-reactive protein are indirect measures of inflammation and probably not of RA disease activity per se. However, an increased AMLR would be expected if inflammation of the joint increased because of the sequestration of lymphocytes and neutrophils to the inflammatory site. Previous investigators have differed over whether the AMLR is correlated with disease activity, but none have conducted longitudinal studies of patients. The study of patients not on any drug treatment, or those on first-line drugs who have not taken medication for at least 48 hours prior to assay, appeared to increase the correlation of the AMLR with the ESR.

Since previous investigators^{14 34-37} have found that autologous serum suppresses the AMLR, possibly because of the proved effect of drugs and their metabolites on the AMLR, it was decided to incubate cells in human pooled AB serum. AMLR patients with Reiter's syndrome were not affected significantly by the addition of autologous serum. Often these patients had only just commenced NSAID therapy, and perhaps this explains the lack of suppression of the AMLR by their serum. This may also explain the much reduced variability of their AMLR compared with that of RA patients. Because the AMLR of patients not on drug treatment was unaffected by autologous serum, the inhibitor in autologous serum is thought not to be an HLA-restricted suppressor factor.

In conclusion because of the demonstration of the AMLR and its correlation with an index of disease activity, this provides evidence in support of the AMLR as an in-vivo phenomenon important in the pathogenesis of RA. This study also suggests that the AMLR may be a useful index and predictor of

future disease activity. The AMLR may provide a useful in-vitro model for the screening of various chemotherapeutic agents in RA.

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