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Antibody penetration of viable human cells. I. Increased penetration of human lymphocytes by anti-RNP IgG

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SUMMARY

Antibody penetration of viable cells and interaction with intracellular antigens may have major consequences for immunopathological processes in connective tissue diseases. We have reported previously that antibody can penetrate viable human lymphocytes. To assess further the role of antinuclear antibodies in this process, peripheral blood lymphocytes (PBMC) were incubated with FITC-conjugated IgG fractions from sera containing anti-RNP (anti-RNP IgG), Ro(SS-A), La(SS-B) and dsDNA antibodies and control sera for 24 h. Using crystal violet to quench cell surface staining, intracellular fluorescence of viable lymphocytes was quantified on the flow cytometer. It was noted that anti-RNP IgG entered 46·4 ± 7·2% of lymphocytes which was significantly higher than anti-Ro(SS-A) (29·9 ± 4·1%, $P<0·05$), La(SS-B) (22·0 ± 7·5%, $P<0·01$) IgG and control IgG (28·8 ± 2·1%, $P<0·05$) and not statistically different from anti-dsDNA IgG (32·6 ± 14·3%). Inhibition experiments showed that the increased number of cells penetrated by anti-RNP IgG was a specific process. Time-course studies showed that anti-RNP IgG entry into cells was different from pooled control IgG. With anti-RNP IgG, positive-staining lymphocytes gradually increased in number from 12 to 24 h incubation, whilst with pooled control IgG, the peak was reached within 5 min. Dual staining experiments suggested that whereas both anti-RNP IgG and pooled control IgG entered B and NK cells, anti-RNP IgG also entered T cells. Using IgG $F(ab')_2$ and Fc fragments from either anti-RNP IgG or pooled control IgG to compete with their FITC-conjugated counterparts indicated that the entry of anti-RNP IgG into viable cells appeared to involve both $F(ab')_2$ and Fc fragments, and pooled control IgG depended exclusively on the Fc portion of IgG. Further investigation by incubating anti-RNP IgG with ^{35}S-methionine-labelled monocyte-depleted PBMC (MD-PBMC) suggested that anti-RNP IgG might react with the corresponding antigens either on the cell surface or within the cytoplasm.

Keywords anti-RNP antibodies antibody penetration flow cytometric analysis intracellular fluorescence

INTRODUCTION

Many autoimmune diseases are characterized by the presence of distinct antinuclear antibodies, such as anti-Sm antibody which is specific for systemic lupus erythematosus (SLE) (Tan & Kunkel, 1966; Northway & Tan, 1972; Ma *et al.* 1983), high titre of anti-RNP antibody which appears in mixed connective tissue disease (Sharp *et al.*, 1972; Sharp, 1974; Gilliam & Prystowky, 1977), and anti-Ro(SS-A) and La(SS-B) antibodies in Sjogren's syndrome (Clarke, Reichlin & Tomasi, 1969; Alspaugh & Tan, 1975; Alspaugh, Talal & Tan, 1976; Alspaugh & Maddison, 1979). Most of these antinuclear antibodies are directed against either small nuclear RNPs (snRNPs) or small cytoplasmic RNPs (scRNPs) which have very complex functions in regulation of gene expression. For example, U snRNPs, the antigens of anti-RNP and Sm antibodies, are actively involved in the pre-mRNP splicing process (Zieve & Penman, 1976; Lerner *et al.*, 1980; Chabot & Steitz, 1987). Ro(SS-A) protein, the antigen of anti-Ro(SS-A) antibody, plays a role in control of RNA transport and translation (Bernstein *et al.*, 1984; Woilin & Steitz, 1984), and La(SS-B) protein, the antigen of anti-La(SS-B) antibody, in transport of RNA polymerase III transcripts (Stephano, 1984; Mathews & Francouer, 1984; Chambers *et al.*, 1988). Because of the critical functions of these antigens, it is

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quite possible that if antinuclear antibodies could penetrate viable cells, they might profoundly alter cell behaviour by interaction with corresponding antigens, and thus play a direct role in the immunopathological processes of autoimmune diseases.

According to current dogma, the role of antibody is confined to the extracellular milieu, with materials enclosed in the cytoplasmic membrane being in a privileged site with respect to interaction with antibody. This, however, has recently been challenged. The concept of antibody entry into living cells, was first broached by Gilliam, Smiley & Ziff (1974) and then studied by others (Alarcon-Segovia, Ruiz-Arguelles & Fishbein, 1978a, 1979b; Alarcon-Segovia, Ruiz-Arguelles & Llorente, 1979c; Alarcon-Segovia, Llorente & Ruiz-Arguelles, 1982; Alarcon-Segovia & Llorente, 1983; Okudaira, Yoshizawa & Williams, 1987). Antibody penetration of viable cells is extremely important, as, if proved true, it can open up vast new avenues of research, especially in the pathogenesis of autoimmune diseases. To investigate this issue rigorously, we have previously developed a new method based on flow cytometric analysis and use of crystal violet to quench surface fluorescence. The results have unequivocally demonstrated that antibody does enter viable human lymphocytes (Ma *et al.*, 1987). To study further the mechanisms underlying antibody penetration of living lymphocytes, penetration by IgG fractions from control sera and sera containing four different antinuclear antibodies have been studied. The evidence presented in this paper shows that anti-RNP IgG enters a higher percentage of viable human lymphocytes than IgG fractions from either control sera or sera containing some other antinuclear antibodies. Additionally, whilst anti-RNP IgG and pooled control IgG enter both B and NK cells, anti-RNP IgG also penetrates T cells by a mechanism that is probably mediated by the $F(ab')_2$ rather than Fc portion of the IgG molecule.

MATERIALS AND METHODS

Human peripheral blood mononuclear cells (PBMC) and monocyte-depleted PBMC (MD-PBMC)

Blood was obtained from normal volunteers and the mononuclear cell fraction isolated by centrifugation on a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). After three washes with phosphate-buffered saline (PBS) (CSL, Melbourne, Australia) PBMC were suspended at a concentration of 1×10^7/ml in RPMI 1640 supplemented with 2 mM Glutamine, 20 mM Hepes and 10% heat-inactivated fetal calf serum (FCS) (RPMI-suppl.). MD-PBMC were prepared by depletion of monocytes from PBMC using plastic adherence (Lopez-Karpovitch *et al.*, 1984). Cell viability for MD-PBMC preparations was at least 97% by trypan blue dye exclusion, and fewer than 1% monocytes could be detected in MD-PBMC preparation using CD14 monoclonal antibody (Becton Dickinson Immunocytome Systems), on EPICS C flow cytometer (Coulter Electronics, Hialeah, FL).

Sera and mouse monoclonal antibody

Sera were obtained from normal volunteers and patients with rheumatic diseases. The specificities of anti-RNP, Ro(SS-A) and La(SS-B) antibodies were determined by counter immunoelectropheresis using standards from the ANA Reference Laboratory, Center for Disease Control, USA. Anti-RNP antibody specificity was also confirmed by Western blotting. Anti-dsDNA antibody was detected by DNA binding assay. The sera containing only monospecific antinuclear antibody were used for the studies. Control sera were donated by healthy hospital staff, and in some cases normal human gammaglobulin (CSL) was used as the source of pooled control IgG. Mouse monoclonal anti-RNP antibody (2·73) (Billings *et al.*, 1982) was a gift from Dr S. Hoch, Agouron Institute, USA.

Lymphocytotoxicity test

All sera were screened for cytotoxic antibodies using the standard lymphocytotoxicity test (Mittal *et al.*, 1968). Anti-HLA antibodies were tested against a panel of cells from 59 individuals, 35 of which were T lymphocytes from normal healthy donors while the other 24 cells were B lymphocytes from patients with chronic lymphocytic leukaemia. All of these cells had previously been stored in liquid nitrogen and thawed for use by standard methods. The viability of all cells used for screening exceeded 90%.

IgG fractions, IgG ($F(ab')_2$ and Fc fragments

Whole sera were loaded on to Protein A Sepharose 4B columns (Pharmacia Fine Chemicals), and after extensive washing with 0·1 M phosphate buffer, pH 7·0 (PB), IgG fractions were eluted from the column with 0·1 M glycine-HCl buffer, pH 3·0.

For IgG $F(ab')_2$ fragments preparation, IgG fractions were digested with pepsin (IgG/pepsin = 10/1 w/w) in 0·2 M acetate buffer, pH 4·5, at 37°C overnight and the reactions were terminated by dialysis against PB. Digests were passed through a Protein A Sepharose 4B column followed by an affinity column coated with anti-IgG Fab fragment specific antibody (Jackson ImmunoResearch Lab., Bar Harbor, ME) to remove undigested IgG molecule and Fc′ fragments. The eluted fractions contained only IgG $F(ab')_2$ fragments having no precipitation line against anti-IgG Fc specific antibody by immunodiffusion.

IgG Fc fragment was prepared by papain digestion of IgG (IgG/papain = 10/1 w/w) in PB containing 0·01 M cysteine and 0·002 M EDTA at 37°C overnight. The digest was dialysed against distilled water at 4°C for 24 h with three changes followed by PB overnight. The Fc fragment was absorbed on to a Protein A column and then eluted with 0·1 M acetate buffer. After dialysing against PBS, the eluate was passed through an anti-Fab fragment specific affinity column to remove any remaining Fab fragments and undigested IgG molecules.

FITC conjugation of IgG fractions

IgG fractions were conjugated with FITC (Sigma Chemical Company, St Louis, MO) as described by Johnson (1981). The concentrations of FITC-conjugated IgG were then adjusted to 10 mg/ml (OD 495:OD 280 = 0·82–1·10).

Intracellular fluorescence

Intracellular fluorescence was measured as described previously (Ma *et al.*, 1987). Briefly, 1×10^6 PBMC were incubated with FITC-conjugated human IgG at a concentration of 1 mg/ml in 100 μl RPMI-suppl. at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. After incubation the cells were washed three times with PBS and resuspended in 300 μl RPMI-suppl. Crystal violet (0·1 mg/ml) (BDH Chemical Ltd, Poole, UK, Product No. 34033) was added to the cell suspensions to

quench cell surface fluorescence and the cells were then run immediately on an EPICS C flow cytometer (Coulter Electronics). The intracellular fluorescence of viable lymphocytes was assessed by gating the lymphocyte population on a profile of forward angle light scatter *versus* 90° light scatter using a 525 nm narrow band filter and collection at channel 12.

Specificity of anti-RNP IgG penetration of lymphocytes
PBMC (1×10^6) were pre-incubated with 10-fold molar excess (6×10^{-7} M) unconjugated IgG (10 mg/ml) from either anti-RNP antiserum or pooled control IgG at 37°C for 30 min, and then 6×10^{-8} M FITC-conjugated anti-RNP or pooled control IgG (1 mg/ml) was added for another 24 h incubation in 100 μl RPMI-suppl. The intracellular fluorescence was determined as above.

Antibody penetration of different lymphocyte populations
To investigate anti-RNP IgG entry into different cell populations, a dual staining technique was employed. After incubation with FITC-conjugated IgG for 24 h, 5×10^6 PBMC were washed three times with RPMI 1640. Phycoerythrin (PE)-conjugated monoclonal antibodies to CD3, 4, 8, 20 (Becton Dickinson), or NKH 1 (Coulter Electronics) were then added to cell suspensions and kept at 4°C for 30 min in the presence of NaN_3. Following three washes with RPMI 1640, cells were resuspended in 300 μl RPMI-suppl. The PE-conjugated anti-CD3, 4, 8 and 20, and NKH 1 antibody stained cell populations were sorted on the flow cytometer and then concentrated to 3×10^6/ml. The 0·1 mg/ml crystal violet was added to the sorted cell suspensions and the percentage of FITC-positive cells was ascertained as above.

Time course of anti-RNP IgG penetration of lymphocytes
The dynamics of antibody penetration were studied by adding FITC-conjugated IgG to cell suspensions at various times and the intracellular fluorescence measured as above.

Inhibition of antibody penetration
To assess the relative roles of the $F(ab')_2$ and Fc portions of the molecules in the process of antibody penetration, inhibition studies were undertaken with $F(ab')_2$ and Fc fragments of IgG. PBMC (1×10^6) were pre-incubated with 10-fold molar excess (6×10^{-7} M) unconjugated IgG $F(ab')_2$ fragments (7·5 mg/ml) from both anti-RNP antiserum and pooled control IgG or Fc fragment (3·1 mg/ml) from pooled control IgG at 37°C for 30 min, and then 6×10^{-8} M FITC-conjugated anti-RNP and pooled control IgG (1 mg/ml) were added for another 24 h incubation in 100 μl RPMI-suppl. The intracellular fluorescence was determined as above.

Detection of cell-associated anti-RNP antibody using ^{35}S-labelled MD-PBMC
The cell-associated anti-RNP antibody was studied using ^{35}S-labelled MD-PBMC. MD-PBMC (2×10^7) were incubated with anti-RNP IgG (1 mg/ml) in 2 ml of methionine-free RPMI 1640 (Flow Laboratories,) containing 10% dialysed heat-inactivated FCS and 20 mM Hepes (methionine-free medium) with 200 μCi of ^{35}S-methionine (Amersham International, Amersham, UK) in a humidified incubator with 5% CO_2 at 37°C for 24 h and then cells washed three times with cold PBS containing 2 mM methionine. The cell viabilities after radiolabelling were determined to be over 95% by trypan blue dye exclusion. The cells were lysed and the extract added to 20 μl of packed Protein A beads which, in order to reduce non-specific binding, had been pre-incubated with unradiolabelled cell extract for 30 min at 4°C. The antigen that bound to the antibody *in vitro*, following incubation of viable cells with the antibody was analysed by 15% SDS–PAGE and autoradiography (Laemmli, 1970). Immunoprecipitation of U1 RNP polypeptides from MD-PBMC using both anti-RNP IgG and mouse monoclonal anti-RNP antibody (2.73) was performed according to Lerner & Steitz (1979).

Table 1. Anti-lymphocyte antibodies

Antinuclear antibody	Lymphocytoxicity (%)	Specificity
Anti-RNP	—	
Anti-RNP	—	
Anti-RNP	—	
Anti-RNP	—	
Anti-RNP	—	
Anti-Ro (SS-A)	4·0	HLA A23
Anti-Ro (SS-A)	—	
Anti-Ro (SS-A)	7·0	HLA A23
Anti-Ro (SS-A)	—	
Anti-La (SS-B)	—	
Anti-La (SS-B)	—	
Anti-dsDNA	—	
Anti-dsDNA	—	
Anti-dsDNA	5·0	Undefined weak
Control	—	
Control	—	
Control	—	

RESULTS

Lymphocytotoxic antibodies
Anti-lymphocyte antibodies have been screened and HLA specificities identified. The results are shown in Table 1. Only low levels of cytotoxicity were detected in three out of 17 sera used for antibody penetration experiments. In two of the anti-Ro(SS-A) sera there were weak reactivities restricted to HLA A23 target cells, and in one of the anti-dsDNA sera there was weak reactivity of unidentified specificity. There was no reactivity in the anti-RNP antisera.

Antibody penetration of viable human lymphocytes
IgG fractions were isolated from five individual anti-RNP, four anti-Ro(SS-A), two anti-La(SS-B) and three anti-dsDNA monospecific antibody-containing sera and three control sera, and FITC conjugated. After incubation of normal PBMC with FITC conjugates for 24 h, viable lymphocytes were gated on the flow cytometer using forward angle and 90° light scatter and intracellular fluorescence assessed (Fig. 1). After incubation with anti-RNP IgG, 46·4 ± 7·2% lymphocytes had intracellular fluorescence which was significantly greater than those with IgG from anti-Ro(SS-A) (29·9 ± 4·1%, $P < 0.05$), anti-La(SS-B) antibody-containing sera (22·0 ± 7·5%, $P < 0.01$) and control sera (28·8 ± 3·1%, $P < 0.05$). There was no statistical difference of

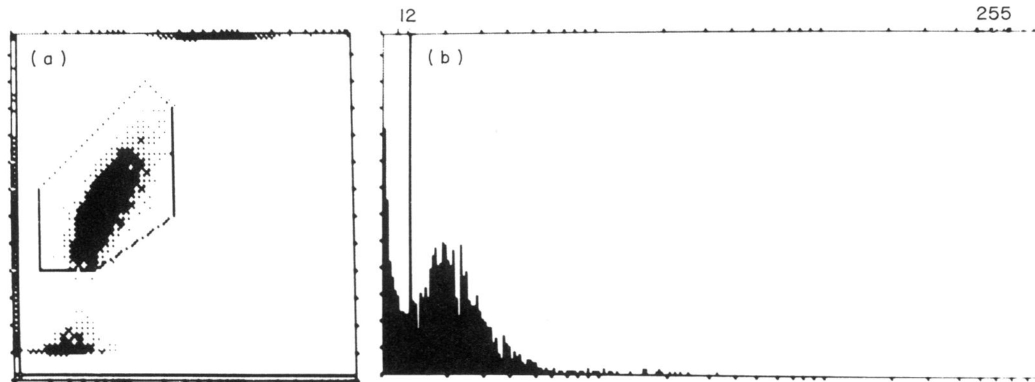

Fig. 1. Flow cytometric histograms of human PBMC incubated with FITC-conjugated IgG from anti-RNP antiserum (1 mg/ml) at 37°C for 24 h after addition of crystal violet (0·1 mg/ml). (a) Plot of forward angle light scatter versus 90° light scatter. The population within the gate is selected for analysis and contains 99% lymphocytes. Dead cells and monocytes are gated out of this plot. (b) Intracellular fluorescence of viable human lymphocytes is collected at channel 12.

Table 2. Antibody penetration of viable human lymphocytes

Antibody	Positive staining (%)	Mean ± s.d. (%)
Anti-RNP	49·2 ± 0·4	
Anti-RNP	53·8 ± 1·6	
Anti-RNP	48·9 ± 1·1	
Anti-RNP	45·4 ± 0·5	
Anti-RNP	34·7 ± 1·1	46·4 ± 7·2
Anti-Ro (SS-A)	29·2 ± 4·5	
Anti-Ro (SS-A)	35·9 ± 1·9	
Anti-Ro (SS-A)	27·7 ± 0·4	
Anti-Ro (SS-A)	26·7 ± 2·0	29·9 ± 4·1*
Anti-La (SS-B)	27·4 ± 0·4	
Anti-La (SS-B)	16·7 ± 0·6	22·0 ± 7·5†
Anti-dsDNA	45·0 ± 1·8	
Anti-dsDNA	17·0 ± 0·2	
Anti-dsDNA	35·8 ± 0·4	32·6 ± 14·3‡
Control	31·2 ± 2·9	
Control	27·4 ± 4·5	
Control	27·7 ± 0·9	28·8 ± 2·1*

* Compared with anti-RNP antibody, $P < 0{\cdot}05$.
† Compared with anti-RNP antibody, $P < 0{\cdot}01$.
‡ Compared with anti-RNP antibody, $P > 0{\cdot}05$.

intracellular fluorescence between either anti-RNP IgG and anti-dsDNA IgG (46.4 ± 7.2 *vs* 32·4 ± 14·3%) or IgG from control sera and anti-Ro(SS-A), La(SS-B), and dsDNA antibody-containing sera (Table 2).

Specificity of anti-RNP IgG penetration of lymphoctes

Anti-RNP IgG and pooled control IgG were utilized to compete with FITC-conjugated anti-RNP IgG entry into viable cells. The results in Fig. 2 show that the proportion of the cells penetrated by FITC-conjugated anti-RNP IgG is inhibited by 47% when PBMC are pre-incubated with unconjugated anti-RNP IgG, whereas it is not influenced by unconjugated pooled control IgG.

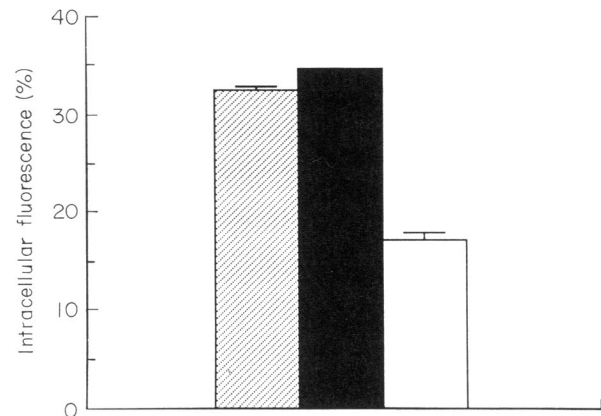

Fig. 2. Intracellular fluorescence of viable human lymphocytes: percentage of stained cells detected by flow cytometry when PBMC were incubated for 24 h with FITC-conjugated anti-RNP IgG alone (■), or after pre-incubation with either unconjugated pooled control IgG (■) or anti-RNP IgG (□).

Antibody penetration of different lymphocyte populations

To investigate anti-RNP penetration of different lymphocyte populations, PBMC were pre-incubated with FITC-conjugated anti-RNP IgG or pooled control IgG for 24 h, then incubated respectively with PE-conjugated anti-CD3, 4, 8, 20 or NKH1 monoclonal antibodies. The flow cytometric histogram of two-colour analysis showed a double stained cell population when PBMC were incubated with both anti-RNP IgG and CD3 monoclonal antibody (Fig. 3a), which was not seen with both pooled control IgG and anti-CD3 antibody (Fig. 3b). To try to analyse FITC-conjugated antibody within different cell populations, PE-conjugated anti-CD3, 4, 8, 20 and NKH1 antibody-positive stained cells were sorted on the flow cytometer and intracellular FITC fluorescence was detected by using crystal violet to quench the surface staining. The results showed that both anti-RNP and pooled control IgG penetrated B (CD20 positive cells) and NK (NKH1 positive cells) cells, but anti-RNP IgG selectively entered 26·0% of T lymphocytes (CD3 positive cells) which was significantly greater than pooled control IgG (5·2%) (Fig. 4). The distribution of anti-RNP IgG in CD4 (22·6%) and CD8 (26·5%) T cell subsets was quite similar.

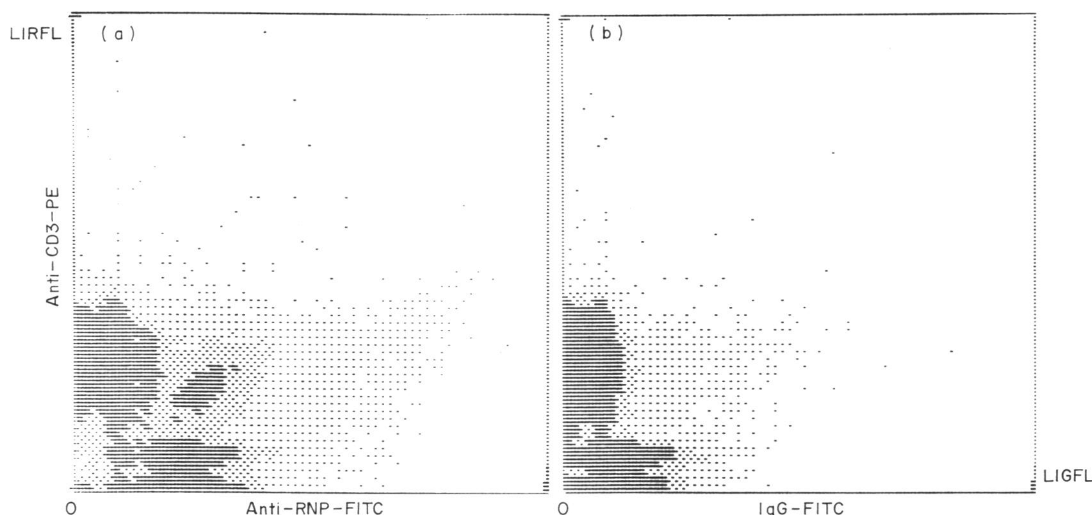

Fig. 3. Two-colour flow cytometric histograms of human lymphocytes incubated with PE-conjugated anti-CD3 monoclonal antibody after incubation with either (a) FITC-conjugated anti-RNP IgG or (b) FITC-conjugated pooled control IgG.

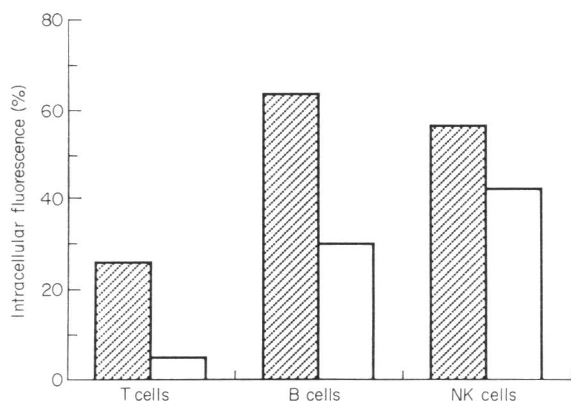

Fig. 4. Percentage of lymphocyte populations penetrated by immunoglobulin. Human lymphocytes were first flow sorted according to the lymphocyte surface phenotypes, using PE-conjugated anti-CD3, CD20 or NKH1 monoclonal antibodies. Intracellular fluorescence of T (CD3 positive cells), B (CD20 positive cells) or NK (NKH1 positive cells) cells was determined when PBMC were incubated for 24 h either with FITC-conjugated anti-RNP IgG ■ or pooled control IgG □.

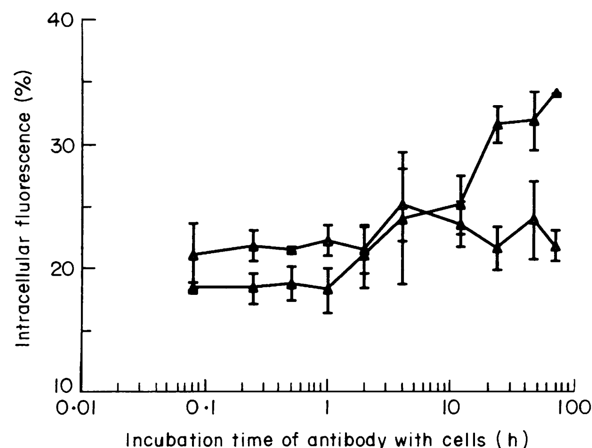

Fig. 5. Time-course studies of antibody penetration. Intracellular fluorescence of human lymphocytes when PBMC were incubated either with FITC-conjugated anti-RNP IgG (▲) or pooled control IgG (△) for 5, 15, 30, 60 min, 2, 4, 12, 24, 48 and 72 h.

Time course of antibody penetration of lymphocytes

The dynamic studies of antibody penetration into viable lymphocytes (Fig. 5) showed that both anti-RNP and pooled control IgG entered living cells at similar rates with incubation time from 5 min to 12 h but anti-RNP IgG penetrated a gradually increasing number of cells from 12 to 72 h. The percentage of sorted T cells penetrated by anti-RNP IgG in 24 h incubation (26·1%) is markedly higher than in 1 h culture (7·9%) whilst no difference can be seen when T cells were incubated with pooled control IgG (6·6 *vs* 5·2%) over this time period.

Inhibition of antibody penetration by F(ab')$_2$ and Fc fragments

Either IgG F(ab')$_2$ fragments from both anti-RNP and pooled control IgG, or Fc fragment from pooled control IgG were used to inhibit antibody penetration *in vitro*. FITC-conjugated anti-RNP IgG entry into viable lymphocytes remarkably reduced when PMBC were pre-incubated with unconjugated F(ab')$_2$ fragments of anti-RNP IgG (40% inhibition) but not those of pooled control IgG. Pooled control IgG Fc fragment could also partially inhibit FITC-conjugated anti-RNP IgG penetration (21% inhibition). Pooled control IgG penetration was only inhibited by IgG Fc portion (47% inhibition) and not by F(ab')$_2$ fragments from either anti-RNP or pooled control IgG (Table 3).

Anti-RNP antibody interaction with cellular RNP antigen of viable cells

To study further the interaction of anti-RNP IgG with viable human lymphocytes, ^{35}S-methionine-labelled MD-PBMC were incubated with anti-RNP IgG and pooled control IgG for 24 h. Recovery of IgG which was associated with ^{35}S-labelled viable MD-PBMC using Protein A, demonstrated that anti-RNP IgG bound to three polypeptides of molecular weights of 34 kD and 29/28 kD (Fig. 6, lane c) but pooled control IgG did not (Fig. 6, lane d). These had the same molecular weights as the U1

Table 3. Inhibition of antibody penetration by IgG F(ab′)₂ and Fc fragments

Inhibitor	Anti-RNP-FITC (%)	Inhibition (%)	Control IgG-FITC (%)	Inhibition (%)
—	32·6±0·2	—	19·5±0·5	—
Anti-RNP F(ab′)$_2$	19·6±0·1	40	17·1±0·7	12
Control IgG F(ab′)$_2$	30·5±0·0	6	7·8±2·1	8
Control IgG Fc	25·7±2·1	21	10·3±1·7	47

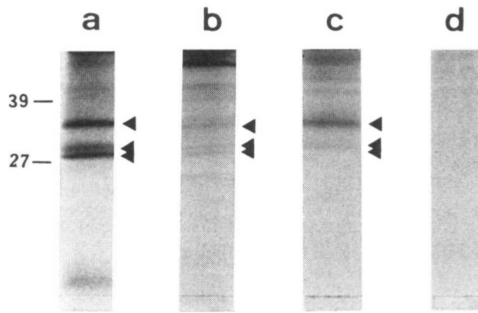

Fig. 6. Anti-RNP IgG binding to cell-associated RNP antigen. Positive controls: U1 RNP polypeptides immunoprecipitated by (a) human anti-RNP IgG or (b) mouse monoclonal anti-RNP antibody (2·73). Cell-associated antigen binding after incubation of MD-PBMC for 20 h with (c) human anti-RNP IgG or (d) human pooled IgG control.

polypeptides A, B′ and B (Luhrmann, 1988) precipitated by both anti-RNP IgG (Fig. 6, lane a) and mouse monoclonal anti-RNP antibody (2.73) (Fig. 6, lane b) on SDS–PAGE. The results suggested that it was the anti-RNP antibody specificity of the IgG preparation, rather than other specificities, i.e. anti-lymphocyte antibody, that had reacted with intracellular and/or cell surface RNP antigen associated with these viable cells.

DISCUSSION

In vitro studies of anti-RNP antibody penetration of living human lymphocytes were first carried out by Alarcon-Segovia and his colleagues and their results suggested that 19% of lymphocytes showed intranuclear fluorescence staining when PBMC were incubated for 1 h with FITC-conjugated fraction from a high-titre anti-RNP antibody-containing serum. They also suggested that the penetration was related to Fc portion of anti-RNP antibody (Alarcon-Segovia *et al.*, 1979a), and that this phenomenon was also seen *in vivo* in 5·5% and 0·2% of lymphocytes in patients with mixed connective tissue disease and SLE respectively (Alarcon-Segovia *et al.*, 1979b). The traditional UV microscopic techniques which they used, however, could neither eliminate false-positive results due to the entry of antibody into dead cells nor morphologically distinguish surface from intracytoplasmic fluorescence, or lymphocytes from monocytes which can pinocytose antibody and comprise 15–30% of PBMC. The evidence of anti-RNP antibody penetrating of lymphocytes presented by Alarcon-Segovia and his colleagues was therefore not sufficiently convincing. In subsequent papers they showed that anti-RNP antibody could abrogate T suppressor function by deletion of one subgroup of T cells and inhibit both RNA and DNA synthesis of these cells (Alarcon-Segovia *et al.*, 1979c, 1982; Alarcon-Segovia & Llorente, 1983). Antinuclear antibody penetration of living lymphocytes was also reported by the others. Okudaira *et al.* (1987) have shown that, after stripping IgG from cell surface, mouse monoclonal anti-DNA antibodies could still be detected within cell extracts when mouse and normal human PBMC were incubated with monoclonal anti-DNA antibodies for a period of over 15 h. Under the microscope, approximately 10% of T lymphocytes showed peroxidase-positive perinuclear staining or actual intranuclear globular staining. They assumed that anti-DNA antibodies might penetrate living cells.

In order to further study antibody penetration of viable lymphocytes, we have established a flow cytometric method to detect intracellular fluorescence and have unequivocally demonstrated that both anti-RNP antibody and control IgG could penetrate viable human lymphocytes (Ma *et al.*, 1987). Dead cells can be distinguished from viable cells because of their lower forward angle light scatter and then excluded by electronic gating on the flow cytometer (Fig. 1a). Our previous studies also showed that 99% of cells which we studied could be positively stained with fluorescein diacetate after 24 h incubation with anti-RNP IgG and, therefore, they were alive (Ma *et al.*, 1987). The present studies extend those observations and show that anti-RNP IgG enters a significantly higher percentage of lymphocytes than either anti-Ro(SS-A), La(SS-B) or control IgG. There was no statistical difference when anti-RNP IgG was compared with anti-dsDNA IgG (Table 2). There are already some data to suggest that anti-dsDNA antibody can penetrate viable lymphocytes by binding to DNA on the cell surface (Okudaira *et al.*, 1987).

Because of tight binding of polyclonal anti-RNP antibody to its antigen, it is extremely difficult to obtain affinity-purified anti-RNP antibody. Additionally, because of the labile nature of RNP antigen, it is virtually impossible to be certain that some of the RNP polypeptides would not disassociate from the column during the elution, accompanying anti-RNP antibody to form immune complexes. We therefore used IgG fraction from sera containing high-titre monospecific anti-RNP antibody to study antibody penetration of viable lymphocytes. The specificity of anti-RNP antibody was confirmed by both immunoelectrophoresis using standard from the Center for Disease Control, USA, and Western blotting. All the anti-RNP IgG formed only one single precipitin line identical to the anti-RNP standard in immunoelectrophoresis and bound to well-recognized U1 polypeptides 70 kD, A or C in Western blotting. No anti-DNA, Ro(SS-A) and La(SS-B) antibodies could be detected by using conventional methods as described in Materials and methods. Participation of anti-lymphocyte antibody has also been ruled out because all anti-RNP IgG lacked any anti-lymphocyte activity (Table 1). In a 10-fold molar excess

concentration, anti-RNP IgG can substantially inhibit the entry of its FITC-conjugated counterpart into living human lymphocytes by 47% (Fig. 2), whilst pooled control IgG does not, suggesting a specific phenomenon that is related to the presence of anti-RNP antibody. The role that the anti-RNP specificity of the IgG preparation played in antibody penetration was further highlighted by using ^{35}S-labelled cells. The results showed that anti-RNP IgG reacted constantly with at least three cell-associated polypeptides of molecular weight 34 and 29/28 kD (Fig. 6, lane c), which had identical molecular weights to U1 polypeptides A, B′ and B precipitated by the same anti-RNP IgG in conventional immunoprecipitation (Fig. 6, lane a), and blotted in Western blotting (data not shown). Mouse monoclonal anti-RNP antibody (2.73) also precipitated these same polypeptides but in lesser amounts in comparison with those precipitated by human polyclonal anti-RNP antibody. This may be because the mouse monoclonal anti-RNP antibody (2.73) only precipitates U1 RNP complex containing the 70 kD U1 polypeptide, whereas human polyclonal anti-RNP antibody also reacts to free polypeptides and partial U1 RNP complex which may not include the 70 kD U1 polypeptide. Precipitation of the 70 kD U1 polypeptide has not been demonstrated; the reasons for this are not clear but may be due to poor metabolic labelling with ^{35}S-methionine. This phenomenon, however, has been commented on by Steitz and her colleagues who noted that the 70 kD polypeptide was only occasionally detectable in anti-Sm or anti-RNP immunoprecipitates (Pettersson *et al.*, 1984). Additionally, most gels in published papers failed adequately to show the 70 kD band because of the non-specific binding in the high molecular weight range of 15% polyacrylamide gel (Matter *et al.*, 1982; Fisher *et al.*, 1983; Reeves *et al.*, 1986). Our data so far suggest that the anti-RNP specificity of the IgG preparation may be responsible for antibody penetration. The structure of the cell-associated antigen bound to anti-RNP antibody *in vitro* is a complicated issue which needs to be investigated further.

Increased penetration by anti-RNP IgG of lymphoid cells is probably due to reaction with a different lymphocyte population from that by pooled control IgG. To be able to demonstrate this, PE-conjugated anti-CD3, 4, 8, 20 and NKH1 monoclonal antibodies were used to phenotype the lymphocytes which have been pre-incubated with FITC-conjugated either anti-RNP or pooled control IgG. On two-colour flow cytometric histogram, a double stained cell population only appeared when PBMC were incubated with both FITC-conjugated anti-RNP IgG and PE-conjugated anti-CD3 antibody, which could not be seen with pooled control IgG (Fig. 3). These results indicated that anti-RNP IgG might selectively penetrate a subset of T lymphocytes. In further studies, lymphocyte populations and subsets were sorted on the flow cytometer according to lymphocyte phenotypes and the intracellular IgG of each cell group then analysed by using crystal violet to quench the cell surface fluorescence. The results again indicated that anti-RNP antibody entered many more T lymphocytes than control IgG (26·0% *vs* 5·2%) and the distribution of anti-RNP IgG in CD4 and CD8 T cell subsets was quite similar, whilst both anti-RNP IgG and pooled control IgG penetrate B and NK cells (Fig. 4). It is well known that B and N cells have larger numbers of Fc receptors on their surface whereas only a small population of T cells bears low affinity Fc receptor (Anderson & Looney, 1986). Although Fc receptor(s) on phagocytes mediate immune complex internalization, the role of Fc receptor(s) on lymphocytes is still not clear. Antibody entry into cells via Fc receptor(s) has been reported by Alarcon-Segovia *et al.* (1979a). The results derived from our studies also suggest that Fc receptor(s) may be involved in IgG internalization because both anti-RNP IgG and pooled control IgG enter B and NK cells. Anti-RNP IgG, however, selectively penetrated T lymphocytes which are known to have few Fc receptor(s) on the cell surface, suggesting that anti-RNP antibody penetration of living lymphocytes might involve other non-Fc-mediated mechanisms.

That a different mechanism exists for entry of anti-RNP IgG is further suggested by time-course studies. Both anti-RNP IgG and pooled control IgG entered a similar proportion of lymphocytes with short incubation periods, but anti-RNP antibody penetrated a gradually increasing number of lymphocytes after 12 h whereas control IgG remained static (Fig. 5). Flow sorting experiment showed that anti-RNP IgG entered many more T cells in 24-h incubation (26·0%) than it did in 1-h culture (7·9%), whereas pooled control entered at a similar rate (6·6 *vs* 5·2%). These results support the hypothesis that some anti-RNP antibody specific binding structures might appear on the surface of a T lymphocyte subset, after incubation with anti-RNP IgG over 12 h, which increases the efficiency of anti-RNP IgG entry into viable T cells. The reason that Fc receptor(s) uptake of anti-RNP antibody was the only stated mechanism in the work of Alarcon-Segovia *et al.* (1979a) is perhaps due to their experimental conditions with only 1 h incubation.

IgG F(ab′)$_2$ fragments generated from both anti-RNP IgG and pooled control IgG, and IgG Fc fragment from pooled control IgG were employed to inhibit antibody penetration of living lymphocytes. The results showed that anti-RNP IgG penetration was specifically inhibited by IgG F(ab′)$_2$ fragments from anti-RNP IgG but not those from pooled control IgG. It was also partially inhibited by IgG Fc fragment, presumably due to blocking of the Fc-mediated uptake of IgG, probably because of the presence of IgG other than anti-RNP specific antibody in anti-RNP IgG preparation. Pooled control IgG was only blocked by IgG Fc fragment (Table 3). These results indicated that anti-RNP IgG penetration was related to both antigen binding site and the Fc portion of IgG molecule whilst control IgG only to Fc fragment.

Non-Fc-mediated antibody uptake has also been noticed by Galoppin & Saurat (1981). They reported that 70% of living keratinocytes showed nuclear speckled staining when incubated with anti-RNP sera and that penetration was not through Fc receptor(s) because only 4% of keratinocytes were able to form rosettes with antibody-coated erythrocytes in their studies. Some studies have shown that nuclear antigens, such as dsDNA and histones, can be detected on the surface of viable lymphocytes (Moyer, 1979; Rekvig & Hannestad, 1979; Bennett, Gabor & Merritt, 1985; Holers & Kotzin, 1985). RNP antigen has also been found on the surface of UV irradiated cultured human keratinocytes (LeFeber *et al.*, 1984). More interestingly, Holers & Kotzin (1985) reported that after 12 h incubation, some anti-histone monoclonal antibodies could bind to the surface of monocytes but failed to bind to fresh cells. Okudaira *et al.* (1987) also observed that an increase in amount of anti-dsDNA antibody associated with cells was related to the incubation time. They found that IgG associated with cells showed 520 ng/10^6 cells after 3 h of incubation, but more than three times this amount (1800 ng/10^6 cells) after 15 h incubation. Some recent data suggested that cell surface DNA seemed to bind to a 30-kD

membrane receptor which might be responsible for the receptor-mediated endocytosis of DNA (Bennett *et al.*, 1985; Gabor & Bennett, 1984; Bennett *et al.*, 1988).

Although the concept of antibody penetration of viable cells has not been fully defined and the mechanisms for antibody penetration are still uncertain, these studies provide evidence that antibody can enter living cells, that anti-RNP IgG penetrates more lymphocytes than control IgG, and that this is probably due to the binding of anti-RNP antibody to an antigen-like structure on the surface of a T cell subset.

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