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Movement of IgG Receptors on the Lymphocyte Surface Induced by Protein A of *Staphylococcus aureus*

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Summary. Fluorescent protein A of *Staphylococcus aureus* (Fl-SpA) acts on the IgG receptors of guinea-pig lymphocytes (GPL) and human lymphoid cells (Seraphina) to induce a redistribution pattern (multiple spots, patches, expelled material, caps) similar to the membrane staining of some living cells with anti-IgG. No ring staining was observed, which implies that Fl-SpA acts as a multivalent cross-linking agent on IgG receptors. A prozone effect dependent on Fl-SpA concentration was observed.

Fl-SpA staining was not abolished 2 hours after trypsinization of the cells, nor was it completely inhibited by pretreatment of the cells with non-fluorescent SpA. A low percentage of cap-like stained cells was recorded even at 4° or in the presence of sodium azide.

Seventeen per cent of GPL are specifically stained with Fl-SpA whereas with fluorescent anti-guinea-pig gamma-globulin serum 45 per cent of the cells are fluorescent. After immunization of the animals with sheep red blood cells (SRBC) 31 per cent of the GPL became positive with Fl-SpA.

Forty per cent of Seraphina cells were stained, whether fluorescent anti-human gamma chain serum or Fl-SpA were used. It was also shown that the Fab fragment of an anti-human gamma chain preparation partially inhibited the specific staining of the Seraphina cells with Fl-SpA. This suggests that both reagents have a common site of action (IgG receptors).

Fl-SpA did not stain human lymphoid cells which bear IgM receptors (Daudi cells); SpA reacts only with the Fc region of IgG. However, by reacting Daudi cells with an anti-IgM serum (containing IgG antibodies) and afterwards with Fl-SpA, specific staining was achieved. Fl-SpA is therefore recommended as a fluorescent reagent for indirect immunofluorescent staining in which IgG antibodies are used.

INTRODUCTION

Protein A of *S. aureus* reacts with the Fc region of the IgG molecule (Forsgren and Sjöquist, 1966). No reactions with other classes of immunoglobulins (Ig) have been recorded (Sjöquist, Forsgren, Gustafson and Stålenheim, 1967).

The Ig molecules identified on the surface of human and murine B lymphocytes belong

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mainly to the IgM class (Pernis, Ferrarini, Foni and Amante, 1971). However, in some species a significant percentage of surface-positive cells bear IgG (Unanue, Engers and Karnovsky, 1973).

If the receptor IgG is identical to the IgG secreted by the progeny of B lymphocyte, as the concept 'receptor equals product' postulates (Mäkelä and Cross, 1970), the labelled SpA could be used as a macromolecular probe for the characterization and eventually the isolation of surface IgG receptors from B lymphocytes.

In this paper the ability of labelled SpA to induce movement (patch and cap formation) of the IgG receptors on the membranes of the B lymphocytes is presented. Lymphocytes purified from guinea-pig spleen and a human lymphoid cell line bearing membrane IgG were used.

MATERIALS AND METHODS

Cells

Lymphocytes were isolated from the spleens of normal and immunized white guinea-pigs (Duncin–Hartley) and subsequently purified either by a one step sodium metrizoate/Ficoll procedure (Lymphoprep, Nyegaard & Company, Oslo) (Boyum, 1968) or by passing the cells through a cotton wool column (Ling, 1968). The purity of the different preparations of GPL varied between 90 and 95 per cent and the viability, after incubation with the fluorescent reagents, was never below 90 per cent, as estimated by the Trypan Blue method.

The guinea-pigs were immunized by three intraperitoneal injections of 10^9 SRBC each, with 3-day intervals between the injections and a 10-day rest before testing.

Human lymphoid cells from a Burkitt's lymphoma (patient Seraphina) established in a long term culture were used. These cells synthesize small amounts of IgG located mainly if not exclusively on their surface (Klein and Nilsson, unpublished results). The cells were maintained in the presence of human skin fibroblasts as feeder cells in F-10 medium (Grant Island Biological Company, New York) with 10 per cent calf serum and antibiotics (100 i.u. penicillin and 50 μg streptomycin/ml). The viability was never below 80 per cent after incubation with the fluorescent reagents.

Daudi cells derived from another patient with Burkitt's lymphoma, which have IgM on their membrane (Klein, Eskeland, Inoue, Strom and Johansson, 1970) were used in control experiments.

Labelling of SpA

SpA from Cowan I organisms was purified by affinity chromatography on an IgG–Sepharose 4B column (Hjelm, Hjelm and Sjöquist, 1972). Fluorescein isothiocyante (isomer I, Sigma, St Louis) was coupled to SpA at a final protein concentration of 10 mg/ml at pH 9·0 in 0·25 M carbonate–bicarbonate buffer (0·05 mg fluorochrome per 1 mg SpA). The mixture was left overnight at 4° and further purified by gel-filtration on Sephadex G-25 equilibrated with phosphate-buffered saline, pH 7·2 (PBS) (Bio-cult Laboratory, Glasgow). The F/P ratio of the various Fl-SpA preparations was between 2·5 and 3·0. The Fl-SpA precipitated with human IgG in a double diffusion test. The precipitation line was fluorescent.

Fluorescent antibodies

Fluorescent rabbit antibody against guinea-pig gamma-globulin (Behringwerke, Marburg) (Fl-anti-GPG) and fluorescent rabbit antibody against human gamma chain (Fl-anti-IgG) or IgM (Fl-anti-IgM) (Dakopatts, Copenhagen) were used.

Antisera and media

Rabbit anti-guinea-pig gamma-globulin serum (Behringwerke) (anti-GPG) and rabbit anti-human gamma chain (anti-IgG) or anti-IgM sera (Dakopatts, Copenhagen) were used. From the rabbit anti-IgG serum Fab fragments were prepared by papain hydrolysis and affinity chromatography of the digest on SpA-Sepharose (Hjelm *et al.*, 1972). Sterile PBS and F-10 media, without serum, were prepared.

Fluorescent staining

Direct and indirect immunofluorescent staining was carried out on living cells. The Seraphina or Daudi cells were washed three times with F-10 without serum. A suspension of 5×10^6–10^7 cells/ml in PBS was prepared. In the direct staining 25–50 μl of this suspension was incubated with 25–50 μl of fluorescent reagent at concentrations indicated below. The temperature and the time of incubation are also specified below. After incubation the cells were washed three times with cold PBS and finally resuspended in 25–50 μl PBS containing 20 mM sodium azide. A drop was placed on a glass slide and covered with a coverslip which was sealed with paraffin oil. Alternatively the cells were left to adhere to the glass slide for 5 minutes, the buffer was sucked off and the preparations were fixed in ethanol–acetone (1 : 1) for 30 minutes and mounted in glycerol–PBS. No significant differences were found between the two techniques. The slides were immediately examined with a Leitz ultraviolet microscope equipped with an HBO-200 mercury lamp. Photomicrographs were taken using high speed Ektachrome film. 200–300 cells were examined and scored in each preparation. In the indirect staining the cells were first incubated with the non-fluorescent reagent, washed three times with cold PBS and reincubated with the fluorescent reagent.

RESULTS

Fl-SpA stained the GPL (Fig. 1) in the same manner as reported for fluorescent anti-Ig sera and mouse living lymphocytes (Raff and Petris, 1973); multiple tiny spots (points), patches (broken rings), caps and some expelled fluorescent material could be seen.

The percentage of specifically stained non-immunized GPL varied between 13 and 21 per cent, with an average, for five animals, of 17 per cent. The Fl-SpA concentration was 1 mg/ml, the temperature was kept at 4° and the incubation lasted for 1 hour. Approximately the same values were obtained when the temperature was raised to 25°. The Fl-anti-GPG antibody stained 35–50 per cent of the cells with an average, for five animals, of 45 per cent (with non-diluted fluorescent antibody). This discordant result could be explained if the Fl-anti-GPG also contained anti-IgM antibodies which stained lymphocytes bearing IgM receptors. On reacting the Fl-anti-GPG with guinea-pig serum in the Ouchterlony test a second fluorescent line (IgM?) beside the IgG line, was seen.

It is reported that a transient shift in lymphocyte receptor Ig class from IgM to IgG is induced by immunizing mice with SRBC (Pierce, Asofsky and Solliday, 1973). The per-

centage of Fl-SpA-positive cells increased in animals thus immunized from 17 per cent to 31 per cent (average for three animals) while the Fl-anti-GPG treated cells showed an increase from 45 per cent to 78 per cent (average for two animals).

Although it has been claimed that 90 per cent of DNP-binding cells in the guinea-pig bind fluorescent anti-gamma$_2$ antibodies (Paul and Davie, 1971) this does not necessarily imply that the Ig molecules on the surface of guinea-pig B lymphocytes belong predominantly to the IgG class (Unanue *et al.*, 1973). Our results clearly show that in the spleen

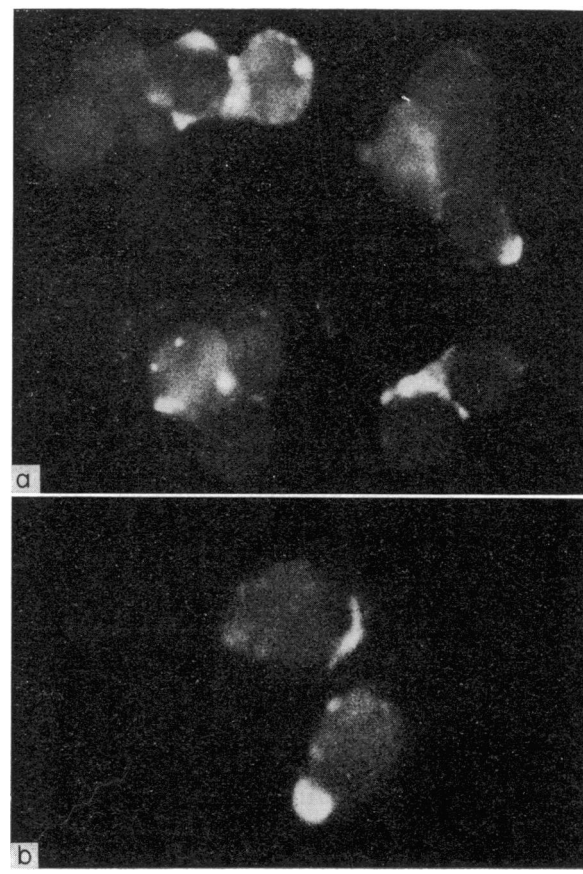

FIG. 1. Fluorescent staining of GPL with Fl-SpA (1 mg/ml). (a) Cells with patches and multiple spots (4°). (b) Cells with cap and expelled fluorescent material (25°). (Magnification × 1400.)

only 20 per cent of the lymphocytes bear IgG class receptors, so that DNP-binding cells probably belong mainly to the IgG-bearing subpopulation.

The percentage of GPL stained with Fl-SpA was 50 per cent less in preparations purified by the cotton wool technique than in those purified by the sodium metrizoate/Ficoll procedure. The increase in the percentage of positive cells during purification was directly proportional to the percentage of lymphocytes in the preparations.

The lower percentage of Fl-SpA stained cells obtained by the cotton wool technique compared with that using the sodium metrizoate/Ficoll procedure suggests that some

IgG-bearing lymphocytes were retained on the cotton wool, as well as macrophages. The macrophages did not constitute a major proportion of the positive cells because lowering their numbers by different purification steps *increased* the percentage of positive cells. Moreover, macrophages have a characteristic intracellular reddish autofluorescence and a distinctive size and could thus be excluded from the scoring of positive cells.

If GPL were first treated at 4° for 1 hour with non-fluorescent anti-GPG serum and then with Fl-SpA (2 mg/ml), about 45 per cent of the cells displayed specific fluorescence (the same percentage as after direct staining with Fl-anti-GPG serum).

Preliminary incubation of GPL with non-fluorescent SpA (1 mg/ml, 4°, 1 hour) reduced the subsequent staining with Fl-SpA performed under the same conditions by about 70 per cent.

The findings by fluorescent staining are summarized in Table 1.

TABLE 1

SUMMARY OF FLUORESCENT STAINING OF GPL

Treatment	Staining conditions		Number of animals*	Percentage of postive cells
	Fluorescent reagent	Concentration (mg/ml)		
Normal	Fl-SpA	1·0	5	17
	Fl-anti-GPG	Non-diluted	5	45
Immunized	Fl-SpA	0·5–1·0	3	31
	Fl-anti-GPG	Non-diluted	2	78
Normal, cotton wool purified	Fl-SpA	1·0	2	8
	Fl-anti-GPG	Non-diluted	1	28
Normal, pretreated with non-diluted anti-GPG (non-fluorescent)	Fl-SpA	2·0	2	45
Normal, pretreated with 1–5 mg/ml of SpA (non-fluorescent)	Fl-SpA	1·0	2	5

* Each experiment was performed at least in duplicate at 4°.

When Seraphina cells were treated with Fl-SpA typical membrane staining of the surface was obtained (Fig. 2).

The percentage of specifically Fl-SpA-stained Seraphina cells varied between 26 and 39 per cent with an average for four experiments of 35 per cent. The percentage of stained cells was dependent on the Fl-SpA concentration, the optimal concentration being 0·6 mg/ml (Fig. 3). In agreement with the Fl-SpA results, the percentage of positive Seraphina cells, after staining with Fl-anti-IgG was 37 per cent (average of three experiments). The percentage of positively stained cells with Fl-SpA was reduced from 38 per cent to 15 per cent by pretreating the Seraphina cells with the Fab fragment of non-fluorescent anti-IgG antibody.

The similarity between the percentage of Seraphina cells which were positively stained with Fl-SpA or Fl-anti-IgG and the partial inhibition of Fl-SpA staining with the Fab fragment of anti-IgG antibody argue for a common site of action for both reagents.

However, one might wonder why only 40 per cent of the Seraphina cells have IgG receptors while, for example, 95 per cent of the Daudi cells possess IgM receptors. The

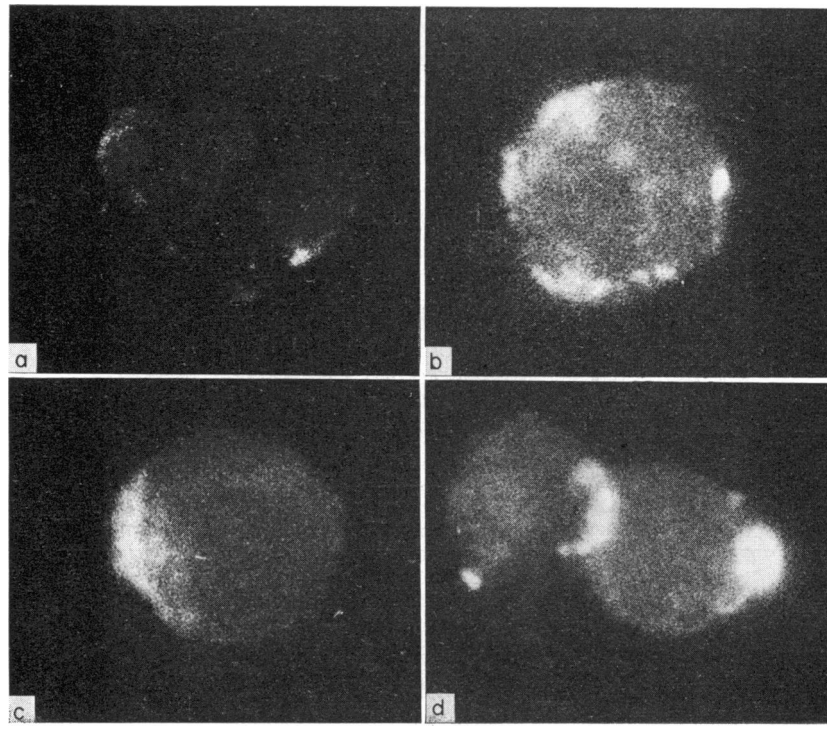

FIG. 2. Pattern of fluorescence in Seraphina cells incubated with Fl-SpA (0·6 mg/ml). (a) Multiple spots. (b) Patches. (c) Caps. (d) Expelled fluorescent material. (Magnification × 3000.)

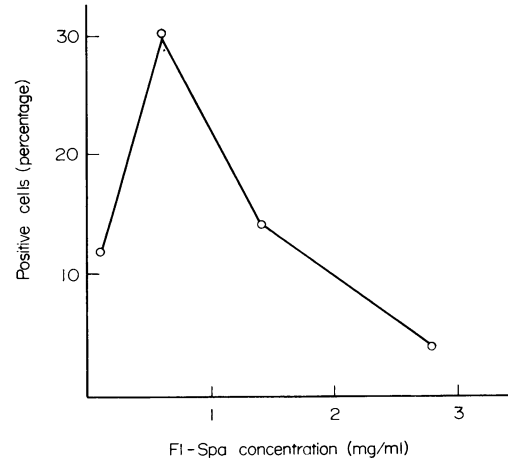

FIG. 3. Effect of Fl-SpA concentration on the percentage of stained Seraphina cells (4°, 1 hour).

values obtained with Fl-SpA were confirmed in a cytotoxicity assay with SpA (1 mg/ml) and rabbit complement, where no more than 30–40 per cent of the cells were killed. Moreover 30 per cent of the activity in a population of ^{125}I-labelled Seraphina cells was retained on an IgG–Sepharose 4B column.

The lack of IgG receptors on 60 per cent of Seraphina cells could be explained if we assume that the thickness of the cell coat, and hence the accessibility of the receptors (Santer, Cone and Marchalonis, 1973), varies in the different phases of the cell cycle, as has been shown for a synchronized human lymphoblastoid line (Rosenfeld, Paintrand, Choquet and Venuat, 1973).

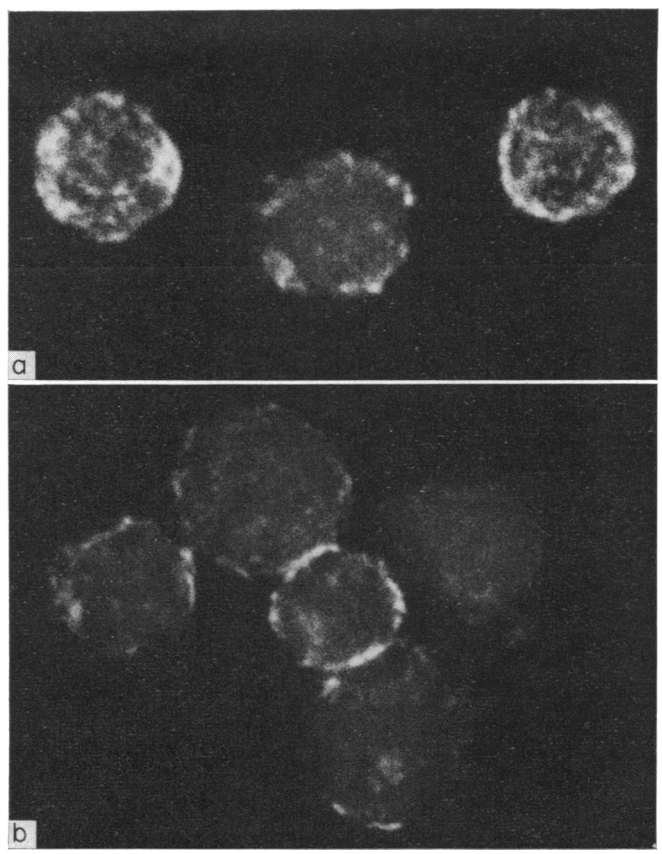

FIG. 4. Fluorescent staining of Daudi cells. (a) Treated with Fl-anti-IgM (4°). (b) Treated with non-fluorescent anti-IgM and afterwards with Fl-SpA (2 mg/ml) (4°). (Magnification ×2000.)

After 2 hours of trypsinization the percentage of positive Seraphina cells was reduced by 70 per cent with both Fl-SpA and Fl-anti-IgG antibody. Twenty-three hours after the trypsin treatment no differences in the percentage of positives between trypsin-treated and untreated cells were recorded.

A preparation of Seraphina cells pretreated with non-fluorescent anti-IgG serum and subsequently incubated with Fl-SpA (1 mg/ml) at 4° for 1 hour showed almost the same

percentage of positive cells (40 per cent) as that found after direct staining with Fl-SpA (35 per cent) or Fl-anti-IgG (37 per cent). Some cells treated in this way showed a ring-type fluorescence not seen in direct staining with Fl-SpA.

As with guinea-pig cells, preliminary incubation of Seraphina cells with non-fluorescent SpA (1 mg/ml, 4°, 1 hour) reduced subsequent staining with Fl-SpA performed under the same conditions by about 70 per cent. Inhibition was obtained despite modification of the SpA/Fl-SpA ratio from 1 : 1 to 5 : 1.

The percentage of Fl-SpA-stained Seraphina cells which presented a cap-like appearance over one pole of the cell after incubation at 25° for 1 hour was only 30–35 per cent of the total stained cells. The same value was obtained if the cells were incubated first with the optimal amount of Fl-SpA (0·6 mg/ml) at 4° for 1 hour and, after washing, were warmed

TABLE 2

SUMMARY OF FLUORESCENT STAINING OF SERAPHINA AND DAUDI CELLS

Treatment	Staining conditions		Number of preparations*	Percentage of positive cells
	Fluorescent reagent	Concentration (mg/ml)		
Seraphina cells, non-treated	Fl-SpA	0·6	4	35
	Fl-anti-IgG	Non-diluted	3	37
Pretreated with non-diluted anti-IgG (non-fluorescent)	Fl-SpA	0·6	2	40
Pretreated with 1 mg/ml SpA (non-fluorescent)	Fl-SpA	0·6–1·2	2	10
Pretreated with 2 mg/ml Fab anti-IgG (non-fluorescent)	Fl-SpA	0·6–1·2	1	15
2 hours after treatment with trypsin	Fl-SpA	0·6	1	10
Daudi cells, non-treated	Fl-SpA	0·6	2	1
	Fl-anti-IgM	Non-diluted	2	95
Daudi cells pretreated with non-diluted anti-IgM (non-fluorescent)	Fl-SpA	0·6–1·2	2	90

* Each cell preparation was examined at least in duplicate at 4°.

to 25°. If sodium azide (20 mM) was included in the incubation medium at 25° 10 per cent of the total stained Seraphina cells still displayed a typical cap-like fluorescence. This was also observed at 4°, almost 10 per cent of the total stained cells displaying caps. The same behaviour was noted with the GPL. In contrast, very few cells with cap-like appearance were seen with the fluorescent antibody at 4° or at 25° in the presence of sodium azide.

Fl-SpA and Fl-anti-IgG did not stain the Daudi cells whereas the Fl-anti-IgM specifically stained more than 95 per cent of them. If the Daudi cells were treated first with non-fluorescent anti-IgM serum (at 4°, 1 hour) and afterwards with Fl-SpA (2 mg/ml, under the same conditions) more than 90 per cent of the cells became fluorescent (Fig. 4).

Results with Seraphina and Daudi cells staining are summarized in Table 2.

DISCUSSION

Fl-SpA induces a redistribution of surface IgG receptors on guinea-pig and human lymphoid cells, causing them to aggregate in numerous spots and/or large patches which then move to one pole of the cell giving a cap-like appearance. The fluorescent staining with Fl-SpA was in many respects similar to that produced by fluorescent antibody against IgG receptors, and resembled that reported for human (Pernis *et al.*, 1971) or mouse (Raff, 1970; Raff and Petris, 1973) living lymphocytes treated with appropriate anti-Ig fluorescent antibody.

However, the Fl-SpA staining has some peculiar features.

(1) *No ring type staining was observed*

Fl-SpA seems to cross-link the receptors in a two-dimensional lattice. The patchy distribution of receptors and their capping require a divalent binding of the fluorescent antibody since fluorescein-labelled monovalent Fab anti-mouse Ig failed to induce patches; instead the cells showed a continuous ring staining (Petris and Raff, 1973). The molar ratio between SpA and human IgG in precipitates, at equivalence, is 1 : 2 (Sjöquist, Meloun and Hjelm, 1972). SpA is thus at least divalent in respect to IgG, and might act as a multivalent cross-linking agent, aggregating IgG receptors which are then observed as multiple spots and large patches.

In accordance with this interpretation, ring-like staining of GPL was obtained when a monovalent fluorescent fragment of SpA (mol. wt 7000) was used in one experiment.

(2) *The cap-like appearance of a small percentage of stained cells at low temperature or at* 25° *in the presence of* 20 mM *sodium azide*

The caps could be artefacts, due to aggregated fluorescent conjugates non-specifically adsorbed on a limited area of the cell surface. However, this phenomenon was never observed with Daudi cells.

Patch formation is a consequence of lattice formation by Fl-SpA or fluorescent antibody with IgG receptors. It takes place at 4° or in the presence of sodium azide (Raff and Petris, 1973), when limited movement and subsequent discrete distribution of the mobile IgG units in the membrane is still possible. If the fluorescent probe has a high affinity for the receptor, or is acting on some crucial submolecular site of IgG we can also imagine that for a few cells the movement and distribution of the receptors could be modified to give a cap-like appearance at 4°.

(3) *The lack of complete inhibition of Fl-SpA staining after previous treatment with non-fluorescent SpA*

This was confirmed indirectly by the results of trypsin treatment and could be understood by assuming that IgG receptors have a high rate of turnover on GPL or on Seraphina cells, since 30 per cent recovery was obtained 2 hours after trypsinization.

The degree of exposure of the cell surface gamma chain, as revealed by Fl-SpA, appears to be such that the C_H2 domain—the probable site of SpA–IgG interaction (Kronvall and Frommel, 1970)—is accessible.

(4) *Dependence of the percentage of positive cells on the concentration of Fl-SpA*

Taylor, Duffus, Raff and Petris (1971) have shown that cap formation is dependent on the concentration of fluorescent anti-Ig (prozone effect). However, the prozone effect may

be more correctly related to patch formation rather than capping if the former is dependent on lattice formation and the latter on membrane movement. Consequently a prozone effect will reduce the size of patches making visualization more difficult and lowering the percentage of stained cells. However, the lower percentage of stained cells at higher Fl-SpA concentrations (Fig. 3) does not mean that in these cases less Fl-SpA is fixed per cell. On the contrary, we have shown with ^{125}I-labelled SpA that at high concentration of SpA more protein is fixed per cell. Apparently visualization of fluorescent cells is hindered by lack of micro-aggregation of the fluorescent material in the lattice.

(5) *The capacity of Fl-SpA to bind to IgG antibodies fixed on cells*

Fl-SpA staining of Daudi cells after a preliminary treatment with a non-fluorescent anti-IgM serum has shown that Fl-SpA cross-links the available Fc region of the fixed antibody. The satisfactory fluorescence intensity recorded recommend Fl-SpA as a reagent for indirect immunofluorescent staining, in place of fluorescent anti-Ig sera. Fl-SpA is only suitable, of course, for visualization of IgG class antibodies.

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