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ON THE MECHANISM OF 'ROSETTE' FORMATION IN HUMAN AND EXPERIMENTAL THYROIDITIS

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SUMMARY

A method of immunocyto-adherence using circulating lymphocytes and sheep red cells coated with an antigen (rosette-formation) was studied in an attempt to detect lymphocytic antibodies. Human and experimental immune thyroiditis were chosen as a model. Such lymphocytic antibodies correlate better with the presence of immune thyroiditis than do humoral antibodies.

The principal aim of the present study was to try to explain the significance of the rosette-forming lymphocyte. The different experiments carried out for instance with the addition of an immunosuppressive drug or trypsin *in vitro* suggest that this lymphocyte may be representative of delayed type hypersensitivity. Other experiments allowed us to think that this cell was unable to passively fix circulating antibodies.

INTRODUCTION

Zaalberg (1964) and Nota *et al.* (1964) described a phenomenon that Nota *et al.* called 'immunocyto-adherence'. Mice and rabbits were hyperimmunized with sheep red cells and it was shown that the spleen and lymph node cells were able to agglutinate the sheep red cells and to form clusters or 'rosettes'. The mechanism was later studied in greater detail by Biozzi *et al.* (1966, 1967, 1968).

It is now well established that the circulating lymphocyte is an immunocompetent cell (Hulliger & Sorkin, 1963; Landy *et al.*, 1964). Taking these facts into consideration, since 1964, this phenomenon of rosette formation was applied to the detection of antibodies which might be of clinical interest, for instance in drug or tuberculin allergy (Frei, Cruchaud & Vannotti, 1965; Frei & Cruchaud, 1966; Cruchaud & Frei, 1967). In this application the circulating lymphocyte is isolated and incubated with sheep erythrocytes which are not actual antigen, but the carrier coated with the suspected antigen.

The mechanism of rosette formation is not yet well understood. Three hypotheses may

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be discussed: (1) Is the rosette-forming lymphocyte the vector of delayed type hypersensitivity? (2) Is this cell in the process of liberating a circulating antibody which has just been synthesized? or (3) has this cell passively fixed such an antibody?

In order to try to answer these questions the following experiments were carried out and will be reported in the present paper: (1) Attempts to block rosette formation by adding an immunosuppressive drug to the incubation medium, (2) attempts to block rosette formation by addition of trypsin, and (3) attempts to produce rosettes with normal lymphocytes previously coated with antibodies.

Human and experimental thyroiditis have been chosen as a model. In autoimmune diseases, circulating antibody titration is known to be of little diagnostic value. Such antibodies have often been demonstrated in all kinds of so called immune diseases; they generally appear as the result rather than the cause of the alteration of the organ; their pathogenic role has been rarely demonstrated.

On the other hand, experimental immune thyroiditis could not be transferred by serum alone. On one occasion the disease was transferred to normal recipients by lymphocytes from donor guinea-pigs (Felix-Davies & Waksman, 1961) and skin tests, which are likely to be cell mediated, were found to be positive (Rose & Witebsky, 1959). Moreover, immunologically activated lymphoid cells were found to be directly cytotoxic for thyroid cell cultures (Rose *et al.*, 1963). For these reasons this condition appeared to us to be the best clinical as well as experimental model for studying the rosette phenomenon. Moreover, this is also an example of delayed type hypersensitivity, similar to our previous models and in this respect different from what was studied by Zaalberg (1964) and Biozzi *et al.* (1966, 1967, 1968).

MATERIALS AND METHODS

(1) *Preparation of thyroglobulin*

The thyroglobulin used as an antigen was extracted from human thyroids by successive $(NH_4)_2SO_4$ precipitations and dialysed against a phosphate buffer, according to the method described by Derrien, Michel & Roche (1948) and Ui *et al.* (1961).

The purity of the product was established by polyacrylamide electrophoresis and by sucrose gradient ultracentrifugation.

(2) *Immunocyto-adherence experiments*

The technique has already been described in detail (Frei & Cruchaud, 1966; Cruchaud & Frei, 1967), so that only the main features are given here.

Preparation of lymphocytes. Twenty millilitres of human defibrinated blood were mixed with 3% gelatin (3 parts of blood, one part of gelatin), according to Coulson's method (Coulson & Chalmers, 1964). Eighty to 90% of the cells recovered from the supernatant were found to be lymphocytes. Five million of these lymphocytes were then suspended in a final volume of 1 ml Hanks's solution. Rabbit lymphocytes were separated with a 3% dextran solution (molecular weight = 250,000) instead of gelatin. Two parts of this solution were mixed with one part of defibrinated rabbit blood. The supernatant contained also about 90% lymphocytes.

Preparation of sheep erythrocytes. Samples of 0·125 ml of packed red cells were formalinized and tanned (tannic acid 1:20,000). One millilitre of that suspension was incubated with 2·5 mg of the thyroglobulin antigen dissolved in phosphate buffer. Samples of 20×10^6 erythrocytes were prepared.

Incubation of sensitized erythrocytes with lymphocytes. Each suspension of 20×10^6 erythrocytes was pooled with a sample of 5×10^6 lymphocytes. Sheep red cells sensitized with human serum albumin (HSA) were used as controls. Readings were made in a counting chamber after 2 hr incubation on a rotating mixer though in a few instances the samples were read after 15 min only. In each case, 2000 white cells were counted. The number of clusters seen was expressed per 1000 white cells. Experience derived from other models allowed us to consider as positive a result greater than 3‰, provided the control was less than 1‰. Exceptional results of 2·0‰ were considered as positive if no rosettes were seen in the control.

(3) *Passive haemagglutination*

The technique described by Stavitsky (1954) was used.

(4) *Addition of an immunosuppressive drug in vitro*

To the pooled erythrocyte–lymphocyte suspension prepared as previously described, 1 or 2·5 mg methotrexate (kindly supplied by Cyanamid International Corporation, Zürich) was added. Readings were made after 2 hr, exceptionally after 15 min. The viability of the lymphoid cells has been assessed by trypan blue exclusion.

(5) *Trypsinization*

Trypsin Difco was diluted 2:1000 in Tris–NaCl buffer, with addition of KCl, Na_2HPO_4, streptomycin and penicillin. Five million lymphocytes were incubated at 37°C with 0·25 ml of this trypsin solution for 30 min. After washing the lymphocytes, the sensitized red cells were added to the medium and the experiment was conducted as before.

(6) *Passive fixation on lymphocytes*

Sera from six patients with anti-thyroglobulin titres between 1:50,000 and 1:1,280 were decomplemented for 30 min at 56° and diluted 1:10. One millilitre of each of these sera was absorbed on 0·2 ml formalinized sheep red cells and 0·3 ml fresh human red cells overnight in the cold, to avoid subsequent red cells agglutination.

One millilitre of each of these sera was incubated for 90 min at room temperature with 5×10^6 lymphocytes from a *normal* donor. The lymphocytes were then centrifuged, washed and pooled with the suspension of thyroglobulin-sensitized red cells. Two hours later clusters were counted as usual in a counting chamber.

(7) *Immunization of the animals*

Experimental thyroiditis was induced in nine random bred rabbits weighing between 2·5 and 4 kg. Twenty milligrams of human thyroglobulin mixed with Freund's adjuvant were injected into the footpads. Three other subcutaneous injections were given on days 8, 16

TABLE 1. Lymphocytic antibodies (number of rosettes) and humoral antibodies (passive haemagglutination titres) in twenty-two cases of human immune thyroiditis

Patient No.	Antigen used for sensitization	No. of clusters/1000 lymphocytes with:		Passive haemagglutination titres
		Antigen-coated red cells	HSA-coated red cells	
1	Thyroglobulin	4·7	0·7	1:2500
	Lung extract	1·7		
2	Thyroglobulin	4·4	1·3	0
	Lung extract	0		
3	Thyroglobulin	3·3	0	1:25
	Lung extract	0		
4	Thyroglobulin	2·5	0	1:250
5	Thyroglobulin	4·7	0·6	1:25000
6	Thyroglobulin	8·5	0·5	1:25000
	Kidney extract	0		
7	Thyroglobulin	8·5	1	1:64000
8	Thyroglobulin	28	5	1:2500
9	Thyroglobulin	3·0	0·5	1:25000
	Muscle extract	0·5		
	Kidney extract	0·0		
10	Thyroglobulin	3·0	0	0
	Kidney extract	0·5		
	Muscle extract	0·5		
11	Thyroglobulin	10	3	0
12	Thyroglobulin	4	0	1:2500
	Kidney extract	0		
13	Thyroglobulin	5·5	0·5	1:25000
	Thyroid extract	5·0		
14	Thyroglobulin	3·0	0	0
15	Thyroglobulin	3·5	0	1:160
16	Thyroglobulin	5·5	2	0
	Thyroid extract	3·5		
17	Thyroglobulin	4·5	1	0
18	Thyroglobulin	3·5	0	0
19	Thyroglobulin	3·5	0	0
20	Thyroglobulin	10·5	2·5	1:2800
21	Thyroglobulin	1·5	0·5	1:25000
22	Thyroglobulin	3.0	0	0

The figures given for lymphocytic antibodies express the proportion of lymphocytes agglutinating sensitized red cells. These data are compared with the figures obtained with HSA-coated red cells used as controls.

and 24. Rosette determination and antibody titration were carried out 8 days after the last injection.

In every case the induction of thyroiditis was controlled histologically.

(8) *Clinical cases*

Five normal donors, twenty-two cases of immune thyroiditis, thirteen cases of hyperthyroidism, eight patients with other diseases of the thyroid were used as lymphocyte donors. The last group was composed of the following diseases: acquired idiopathic hypothyroidism (four cases), toxic adenoma (two cases) and non-toxic goitre (two cases). As a basis for the diagnosis of immune thyroiditis, the following points were considered: rubbery consistency of the gland, clinical evolution, results of thyroid function tests and in some cases histological picture. A clinical study including these patients is being prepared (Scazziga, Perrudet-Badoux & Frei, 1969).

RESULTS

In Table 1 data obtained for lymphocytic antibodies (rosettes) and humoral antibodies (passive haemaglutination) are given and compared with each other. Rosette formation with thyroglobulin as the antigen was shown to be present in twenty-one out of twenty-two cases of immune thyroiditis, while humoral antibodies were found in only thirteen out of twenty-two patients. In three of these cases a saline extract from a human thyroid gland was also used instead of thyroglobulin; lymphocytic antibodies were also demonstrated against this antigen but the number of rosettes was lower. In six cases, antibodies against other kinds of antigens such as kidney, lung or muscle extracts were also looked for; the results were negative.

Results obtained in thirteen patients with hyperthyroidism are reported in Table 2. Lymphocytic antibodies were shown by rosette formation in only one of these patients, whereas humoral antibodies were present in nine cases. In eight patients suffering from other thyroid diseases (Table 3), the results obtained for rosette formation were negative, with the exception of Case No. 1. Two days before this patient was given a therapeutic dose of 18 mc ^{131}I. In none of five controls without thyroid pathology were these two kinds of antibodies found to be positive.

Data obtained by addition of an immunosuppressive drug (methotrexate) into the incubation medium are given in Table 4. In seven experiments carried out with human lymphocytes no change in rosette formation was observed if the drug concentration was 1 mg/ml suspension. However, a striking decrease in the number of rosettes was obtained each time 2·5 mg of the drug was added. This inhibitory action of methotrexate on rosette formation has also been found with other antigens in other conditions such as drug allergy, rheumatoid arthritis, etc.

As shown in ten other cases (Table 5) the addition of trypsin at a low concentration did not appear to alter the number of clusters. The decrease is definite in only one of them.

In the experiments conducted to rule out the hypothesis of a passive sticking of antibodies to lymphocytes, the results showed that even after incubation with high titre anti-thyroglobulin sera, lymphocytes from normal donors did not agglutinate sensitized red cells.

TABLE 2. Lymphocytic antibodies (number of rosettes) and humoral antibodies (passive haemagglutination titres) in thirteen cases of hyperthyroidism

Patient No.	No. of clusters/1000 lymphocytes with:		Passive haemagglutination titres
	Antigen-coated red cells	HSA-coated red cells	
1	0.5	0	0
2	0·0	0	0
3	0·0	0	0
4	0·5	0	1:2500
5	1	0	—
6	0·5	0	1:2500
7	0·5	0	1:640
8	1·5	0·5	0:160
9	0·5	0·5	1:25000
10	0·5	0·0	1:250
11	1·0	1·0	1:2500
12	4·0	1·0	0
13	1·0	1·0	

The figures given for lymphocytic antibodies express the proportion of lymphocytes agglutinating sensitized red cells. The data are compared with the figures obtained with HSA-coated red cells used as controls.

TABLE 3. Lymphocytic antibodies (number of rosettes) and humoral antibodies (passive haemagglutination titres) in eight cases of other thyroid diseases

Patient No.	Diagnosis	No. of clusters/1000 lymphocytes with:		Passive haemagglutination titres
		Antigen-coated red cells	HSA-coated red cells	
1	Toxic adenoma	3·0	0	0
2	Toxic adenoma	1·0	0·5	0
3	Non-toxic goitre	1·0	0·5	0
4	Non-toxic goitre	0·0	0·0	0
5	Hypothyroidism	1·0	0·5	0
6	Hypothyroidism	0·5	0·0	1:20
7	Hypothyroidism	1·0	0·5	0
8	Hypothyroidism	0·5	0·0	0

The figures given for lymphocytic antibodies express the proportion of lymphocytes agglutinating sensitized red cells. These data are compared with the figures obtained with HSA-coated red cells used as controls.

TABLE 4. Influence of methotrexate on the number of clusters, when the drug is added to the incubation medium at two different doses

Patient No.	No. of clusters/1000 lymphocytes		
	Without methotrexate	With 1 mg methotrexate	With 2·5 mg methotrexate
1	3·5	3	0·5
4	2·5	1·5	—
6	8·5	8	3
15	3·5	4·0	0·5
17	4·5	4	0·5
18	3·5	3	0·5
19	3·5	3	0·5
20	10·5	10·5	3·5

Lymphocytes donors are patients from Tables 1 and 3.

TABLE 5. Number of clusters obtained with lymphocytes previously treated with trypsin 2:1000

Patient No.	No. of clusters/1000 lymphocytes with:		
	Antigen-coated red cells	HSA-coated red cells	Trypsin
1	2	0·5	2
2	4·5	0·5	3·5
3	4	1	2
4	5·5	1·5	2
5	4·5	1·5	3·0
6	6·0	1·5	3·0
7	4·5	1·0	3·5
8	9	—	8
9	8	1	6
10	17	2	12

In eight out of nine rabbits hyperimmunized with human thyroglobulin, lymphocytic antibodies could be demonstrated (Table 6 and Fig. 1) (Witebsky & Rose, 1959). In all of them, the serum contained high haemagglutination titres, which may also represent hetero-antibodies. The histological signs of thyroiditis were clearly demonstrated in two rabbits only (rabbits Nos. 5 and 7). The influence of methotrexate added *in vitro* was the same as that observed with human lymphocytes.

In ten experiments with human or rabbit lymphocytes, the preparations were read after 15 min incubation. No rosettes were found at that time.

TABLE 6. Lymphocytic antibodies (number of rosettes) and humoral antibodies (passive haemagglutination titres) in nine rabbits hyperimmunized with thyroglobulin

Rabbit No.	No. of clusters/1000 lymphocytes with:			Passive haemagglutination titres
	Antigen-coated red cells	BSA-coated red cells	2·5 mg methotrexate	
1	4	0	—	1:80 000
2	3	0	0·5	1:40 000
3	3·5	0	0·5	1:10 000
4	2	0	0	1:10 000
5*	12	0	5	1:80 000
6	2	0	1·0	1:160 000
7*	4	0·5	1·0	1:160 000
8	2·5	0	1·0	1:80 000
9	0	0	—	1:80 000

The figures given for lymphocytic antibodies express the proportion of lymphocytes agglutinating sensitized red cells. These data are compared with the figures obtained with BSA-coated red cells used as controls. The last column gives the number of clusters simultaneously counted in a sample to which methotrexate was added.

* Histological evidence of thyroiditis.

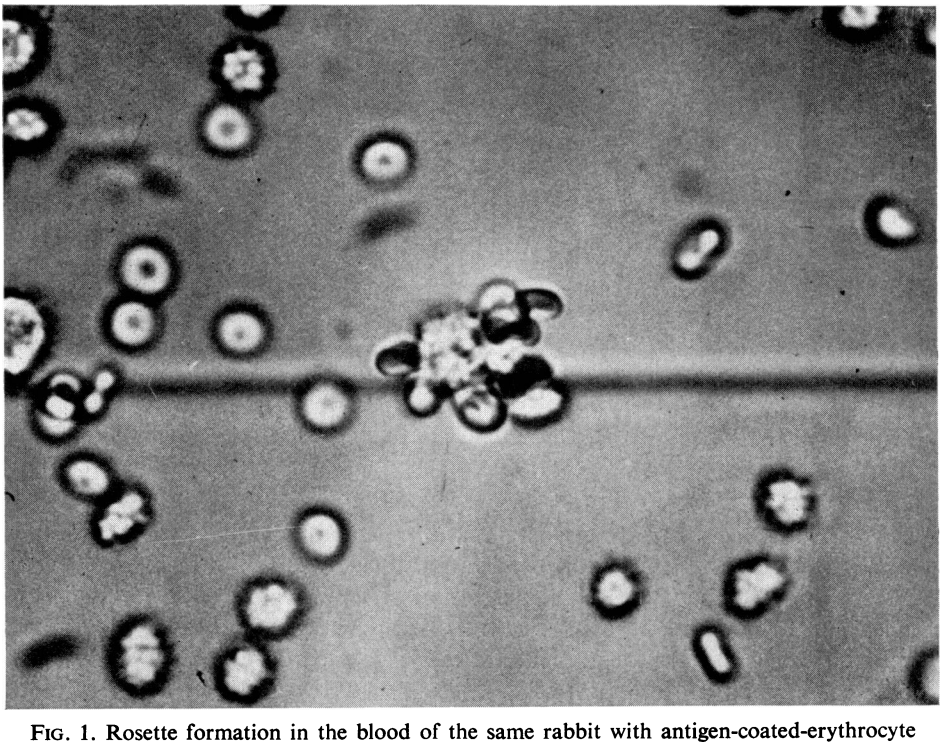

FIG. 1. Rosette formation in the blood of the same rabbit with antigen-coated-erythrocyte rosettes are rarely complete nor do they show a better arrangement than illustrated here. × 1200.

DISCUSSION

As clearly shown in Tables 1, 2 and 3, the clinical picture correlates better with lymphocytic than with humoral antibodies. These results confirm previously published observations (Frei *et al.*, 1968). With one exception immune thyroiditis was accompanied by lymphocytic antibodies as suggested by rosette formation, whereas humoral antibodies were demonstrated by passive haemagglutination in 50% of the cases. On the other hand it is significant that rosette formation was not found in hyperthyroidism nor in other thyroid diseases. The clinical significance will be discussed in another paper (Scazziga *et al.*, 1969 in preparation).

It is established that antibodies demonstrated in the serum by complement fixation, haemagglutination, precipitation or immunofluorescence are in no way specific for Hashimoto's thyroiditis since they are frequently present in many kinds of thyroid disease and also in patients without thyroid disease (Hackett & Forbes, 1960; Hill, 1961). Only a high titre of such antibodies can help to distinguish diffuse autoimmune thyroiditis from other thyroid conditions (Roitt & Doniach, 1958; Ahuja, Tandon & Jayaram, 1966; Scazziga, 1966; Anderson *et al.*, 1967).

In our experiments, it was demonstrated that rosette formation was specific for antigens of thyroid origin. No correlation could be found between the number of rosettes and the stage of the disease. Within the thyroiditis group, no correlation was shown with respect to the functional state of the gland.

Since the experiments of Biozzi *et al.* (1966, 1967, 1968) an important step towards understanding the mechanism of rosette formation was made in an instance of immediate hypersensitivity. Nevertheless, the formation of rosettes by circulating lymphocytes and non-particulate antigens, as in our system, is far from being explained; and our experiments may deal with delayed type hypersensitivity. Two hypotheses can be discussed as basic to the appearance of this phenomenon. In the first one the antibodies could have been produced by other cells than the rosette-forming lymphocyte and could have secondarily stuck to it; this passive fixation was suggested by Bussard (1964). In the second hypothesis the rosette-forming cells themselves could have synthesized the antibodies during the incubation. As an alternative to this idea the lymphocyte could also be considered as a delayed hypersensitivity mediator, even if no immunoglobulin were to play a role in this system.

The results reported in Tables 4, 5 and 6 allow us to discuss the following arguments in favour of this second hypothesis:

(1) It is well known that methotrexate is able to inhibit antibody production since it is an antagonist of folic acid which is necessary for DNA synthesis. Rosette formation is completely suppressed when 2·5 mg/ml of this drug is added to the lymphocyte suspension. The dose of 1 mg is practically unable to block the phenomenon. The presence of this threshold between close doses of 1 and 2·5 mg is rather striking and unexplained. The dose required for '*in vitro*' suppression of rosette formation is much higher than the usual concentration employed in '*in vivo*' immunosuppression. This could be explained by the fact that '*in vitro*' conditions are quite different or also by a direct action of methotrexate on the cell membrane. An inhibition of the adherence between both cell surfaces by a mechanical phenomenon cannot be definitely excluded; nevertheless the addition of the drug into a medium where rosettes are already present has no destructive effect. Although very high, the dose of methotrexate does not kill the cell, as shown by trypan blue exclusion.

(2) The experiment of passive fixation is negative. Normal lymphocytes are unable to form

rosettes even if they have been previously incubated with antibodies. It is probable that the rosette-forming cell cannot be sensitized passively by antibodies, contrarily to what is known for macrophages.

(3) Treatment of lymphocytes by a low concentration of trypsin, before starting the incubation with the red cells, does not prevent them from producing rosettes later, though the number of these rosettes is sometimes decreased (Table 5).

(4) In cases which were positive after 2 hr, a sample taken after 15 min incubation did not contain any rosettes. If the phenomenon was due to passively coated antibodies, it would have been present at that time, since circulating antibodies would have already been fixed to the surface of the lymphocyte.

(5) The absence of any correlation, at least in man, between the number of rosettes and the titre of circulating antibodies speaks against the hypothesis of passive antibody fixation on lymphocytes. Moreover, trypan blue stained cells were never seen in a rosette.

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