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MEMBRANE-ASSOCIATED IMMUNOGLOBULINS OF HUMAN LYMPHOCYTES IN IMMUNOLOGIC DISORDERS

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SUMMARY

Membrane-associated immunoglobulins of peripheral blood lymphocytes were studied by indirect immunofluorescence for γ, α, μ, κ and λ chains in healthy subjects and patients with immunologic disease.

In healthy subjects, heavy chains were found on 30·7% of lymphocytes (γ 15·3%, α 7·2% and μ 8·2%) and light chains on 32·8% of cells (κ 20·4% and λ 12·4%). Patients with humoral immune deficiencies had fewer immunoglobulin-bearing cells; sarcoidosis or thymectomy patients had normal or decreased immunoglobulin-bearing lymphocytes; cells with light chains were fewer than those with heavy chains on their lymphocytes. In some cases, normal levels of serum immunoglobulins were found in the absence of the corresponding immunoglobulin-bearing cells, and in others normal immunoglobulin-bearing lymphocytes were present in the absence of the corresponding serum immunoglobulins.

These data suggest that (1) immunoglobulin-bearing lymphocytes in blood do not reflect the condition of immunoglobulin-synthesizing cells in peripheral lymphoid tissues, and (2) in certain immunologic disorders, either some B-lymphocytes do not synthesize immunoglobulins, or immunoglobulins are in such a situation that the whole molecule or part of the molecule is not visualized by current methods.

INTRODUCTION

Immunoglobulins have been demonstrated on the surface of human lymphocytes and they were studied in a number of pathological conditions by investigators using either the mixed-antiglobulin reaction (Coombs, Feinstein & Wilson, 1969) or a 'sandwich' radioimmunolabelling technique (Wilson & Nossal, 1971), or immunofluorescent staining (Grey, Rabellino & Pirofsky, 1971; Papamichael, Brown & Holborow, 1971; Siegal, Pernis & Kunkel, 1971; Preud'Homme and Seligmann, 1972; Cooper and Lawton, 1972; Fröland and Natvig, 1972; Aisenberg & Bloch, 1972; Piessens *et al.*, 1973). It is widely accepted that immunoglobulin-bearing cells revealed by these methods are thymus-independent or B-lymphocytes.

The results of different studies about direct immunofluorescence of normal peripheral

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blood lymphocytes fall into two groups. Some report about 30% of stained cells, 50% for IgG, 30% for IgM, and 20% for IgA (Cooper, Lawton & Bockman, 1971; Grey *et al.*, 1971; Cooper & Lawton, 1972); others report 10–20% positive cells of which 45–70% stain for IgM, 15–45% for IgG and 10% for IgA (Siegal *et al.*, 1971; Fröland, Natvig & Berdal, 1972; Aisenberg & Bloch, 1972). No satisfactory explanation has been offered to account for these discrepancies in results obtained with similar techniques.

Studying normal human peripheral blood lymphocytes with an indirect or 'sandwich' immunofluorescent technique, we obtained results similar to those of the first category. Patients with immunoglobulin deficiency or cell-mediated immunity deficiency, or both, usually have few immunoglobulin bearing-cells and a disequilibrium between the light (L) and the heavy (H) chains.

MATERIALS AND METHODS

Subjects

Thirty-three individuals were investigated. Thirteen were healthy subjects. Six patients (1–6) had immunoglobulin deficiencies of various sorts and were all incapable of synthesizing antibodies. Eight (7–14) had acute or subacute sarcoidosis, none of whom was under corticosteroid treatment. Five patients had had thymectomy 2 years previously for myasthenia gravis (15 and 16), thymoma (17), parathyroid tumour (18) and thymus hypertrophy (19). One patient (20) had bone marrow aplasia due to chloramphenicol.

Lymphocytes

Lymphocytes prepared from heparinized blood on a Ficoll–Ronpacon gradient (Böyum, 1968), and washed three times, were suspended in cold Eagle's MEM containing 1% foetal calf serum (FCS). Counts of May-Grünwald-Giemsa preparations from normal blood were (mean$\pm$2SD): lymphocytes 91·8% ($\pm$6·0), monocytes 2·9% ($\pm$5·1), polymorphonuclears 5·3% ($\pm$7·0).

Rabbit antisera

Antisera were raised against human IgG, IgA, IgM as well as κ and λ chains.* IgG was purified from a 16% commercial solution of γ-globulin (Croix-Rouge Suisse, Bern, Switzerland) on DEAE cellulose. IgA was isolated from pooled human colostrum using the method of Montreuil *et al.* (1960). IgM was obtained from two patients suffering from Waldenström's macroglobulinaemia with IgM-cryoglobulin. Repeated precipitation in the cold and dissolving in saline at 37°C resulted in pure IgM-globulin. Two κ and two λ Bence-Jones proteins were isolated from urine of patients with multiple myeloma. Thus rabbits were immunized with either purified antigens obtained from pooled normal immunoglobulins or with two paraproteins of the same type obtained from different patients. Anti-IgA was absorbed with transfer piece; anti-IgA and anti-IgM were absorbed with purified IgG, and anti-IgA, -IgM and IgG were made H chain-specific by absorption with κ and λ chains. Anti-κ and anti-λ were absorbed with λ and κ chains respectively as well as with Fc fragments of human IgG. The IgG fraction of each antiserum was isolated by chromatography on

* Some of anti-κ and anti-λ sera and κ and λ Bence-Jones proteins were a gift from Professor S. Barandun, Institut für klinisch-experimentelle Tumorforschung, Bern, Switzerland.

DEAE cellulose. The fractions were adjusted to 6–8 mg protein/ml, and were then reacted with human peripheral lymphocytes to remove anti-HL-A antibodies. For this purpose, one ml of antiserum was absorbed with 40×10^6 lymphocytes at room temperature for 30 min.

Antisera which were then all monospecific on immunoelectrophoresis were ultracentrifuged at 114,000 *g* for 1 hr to remove antigen–antibody complexes that might adhere to lymphocyte membrane (Uhr, 1965). They were divided into small aliquots and stored at −20°C until used.

*Goat antiserum to rabbit Fc fragment**

This antiserum was conjugated with fluorescein isothiocyanate according to Wood, Thompson & Goldstein (1965). IgG fractions of the conjugate were obtained by chromatography on DEAE cellulose by stepwise elution, absorbed with human lymphocytes, ultracentrifuged and stored in the same way as rabbit antisera. The fluorescein: protein ratio was between 2·0 and 4·0. When incubated alone with human lymphocytes, the conjugate did not give any fluorescence.

Determination of optimal concentration and specificity of rabbit antisera

To make sure that rabbit antisera to H or L chains would detect the maximal number of lymphocytes bearing the corresponding chains, dilution and concentration curves were established. 0·02 ml of lymphocyte suspension was incubated with 0·04 ml of undiluted or diluted 1/2, 1/3 and 1/4 antiserum and then with fluorescent goat anti-rabbit Fc fragment (see detailed technique below). The fluorescent lymphocytes were counted and their number was plotted against the different dilutions of antisera. In most cases, there was first a plateau, and then a decrease in the percentage of fluorescent cells; the highest dilution of antiserum still corresponding to the plateau was used subsequently. When no plateau was obtained with diluted antisera, then 0·02 ml of lymphocyte suspension was incubated with either 0·04, 0·08, 0·16 or 0·32 ml of undiluted antiserum and the smallest volume of antiserum giving a plateau value was used for subsequent work.

The specificity of each rabbit antiserum was checked by absorption with the corresponding antigen. Antisera resulting from immunization with pooled normal immunoglobulins (IgG, IgA) were absorbed with the same antigen. Antisera obtained after immunization with two paraproteins (IgM, κ, λ) were absorbed with paraproteins from different sources. Antisera were then assayed (1) on immunoelectrophoresis with normal human serum and with antigens used for immunization, and (2) on lymphocytes. In all cases absorption resulted in the disappearance of specific lines on immunoelectrophoresis and of cell-membrane fluorescence.

Detection of surface immunoglobulins on lymphocytes

Aliquots of 0·02 ml of lymphocyte suspension ($0{\cdot}5 \times 10^6$ cells) were incubated in an ice-bath for 30 min with a volume (usually 0·04 ml) of appropriately diluted IgG from rabbit antiserum to either γ, α, μ, κ or λ chain. They were washed three times, resuspended in medium and incubated for 30 min in the cold with 0·04 ml of fluoresceinated goat anti-rabbit Fc fragment. After four washings cells were suspended in PBS containing 3·5%

* This antiserum was a gift from Dr G. Torrigiani, Immunology Unit, WHO, Geneva, Switzerland.

BSA. A drop of suspension was placed on a slide, overlayed with a coverslip, and the edges were sealed with paraffin. Preparations were kept at 4°C until they were examined. Lymphocytes were identified with light before they were examined for fluorescence. At least 200 lymphocytes were counted on each preparation, in many occasions by two independent investigators.

For each preparation, the number of lymphocytes that were stained following incubation with IgG from normal rabbit serum (0–3% except in three patients) was subtracted from the number of cells stained by any of the antisera.

Lymphocyte cultures

The method of lymphocyte culture was based on that reported by Girard, Poupon & Press (1971). Lymphocytes were isolated on a Ficoll–Ronpacon gradient and were cultured in medium TC 199 (Difco, Detroit, U.S.A.) supplemented with 20% human AB serum. All cultures were done in duplicate.

The following stimulating agents were used: phytohaemagglutinin (PHA-M) (Difco, Detroit, U.S.A.); PPD (Staatenserum Institut, Copenhagen, Denmark); streptokinase–streptodornase (SK/SD, Varidase) (Lederle, London, England); extract of *Candida albicans* (Hollister-Stier, Washington, D.C., U.S.A.). Response was evaluated by the uptake of [^{3}H]thymidine after 3 days of incubation for PHA and 5 days for antigens. It was classified as normal, moderately decreased, or markedly decreased. The response was regarded as normal when lymphocytes were stimulated by PHA and at least by one of the three antigens used; a moderately decreased response (↙) means that lymphocytes were poorly stimulated by PHA but did respond to at least one of the antigens or, alternatively, that lymphocytes were stimulated by PHA but by none of the antigens; the response was classified as markedly decreased (↙↙) when lymphocytes were not stimulated by PHA or any of the antigens.

Determination of immunoglobulin levels in serum

Serum immunoglobulins were evaluated by single radial immunodiffusion, using Tri-Partigen and LC-Partigen plates (Behringwerke, Marburg, Germany) for determination of IgG and IgA, and Immuno-plates (Hyland, Los Angeles, U.S.A.) for the determination of IgM-globulin.

RESULTS

The lymphocytes' fluorescence appeared as uniform rings or, more often, as multiple fluorescent spots located on the cell membrane. Caps were seen only seldom.

Healthy subjects (Table 1)

The sum of lymphocytes reacting with the different anti-H chain antisera averaged 30·7% (range 23–45%); of those, 15·3% (12–19%) reacted with anti-γ serum, 7·2% (4–14%) with anti-α and 8·2% (6–12%) with anti-μ antiserum. 32·8% (17–41%) of lymphocytes reacted with anti-L chains antisera, 20·4% (8–29%) with anti-κ and 12·4% (8–19%) with anti-λ. The κ:λ ratio was 1·6 and the L:H ratio was 32·8:30·7.

Nine subjects had more lymphocytes reacting with anti-L chain than with anti-H chain

TABLE 1. Healthy subjects

Subjects			Ig-bearing lymphocytes							L/H	Serum Ig		
			H chains				L chains						
No.	Sex	Age	γ	α	μ	Total	κ	λ	Total		IgG	IgA	IgM
				(%)				(%)				(mg/100 ml)	
1	M	52	17	5	6	28	8	9	17	17/28		ND*	
2	M	34	14	6	8	28	26	10	36	36/28	1255	294	95
3	F	52	15	7	7	29	28	10	38	38/29		ND*	
4	F	42	16	5	7	28	19	17	36	36/28	1150	210	45
5	F	49	18	7	8	33	15	9	24	24/33	1460	338	82
6	M	41	12	9	9	30	10	16	26	26/30	1140	173	47
7	M	26	19	14	12	45	24	14	38	38/45	1080	327	83
8	F	50	19	6	10	35	29	12	41	41/35	1160	154	95
9	F	42	12	5	8	25	23	8	31	31/25	1180	308	135
10	M	44	13	4	6	23	21	11	32	32/23	910	215	95
11	F	44	14	8	6	28	24	11	35	35/28	1230	273	50
12	M	54	13	6	12	31	19	16	35	35/31	1875	251	100
13	M	27	17	12	8	37	20	19	39	39/37	1740	241	85
Average			15·3	7·2	8·2	30·7	20·4	12·4	32·8	32·8/30·7	1289	253	83
Mean ± 2 SD of 50 controls											1230 ± 395	195 ± 135	126 ± 100

* ND = not done.

TABLE 2. Immunologic disorders

Patients				Ig-bearing lymphocytes							L/H	Serum Ig			Cell-mediated immunity
				H chains				L chains							
No.	Sex	Age	Clinical data and diagnosis	γ	α (%)	μ	Total	κ	λ (%)	Total		IgG (mg/100 ml)	IgA	IgM	
Immunoglobulin deficiency syndromes															
1	M	24	X-linked agammaglobulinaemia	5	2	1	8	7	3	10	10/8	26	6	0	N
2	M	3	X-linked agammaglobulinaemia	0	0	0	0	7	3	10	10/0	132	0	0	N or ↙
3	M	54	Late onset agammaglobulinaemia	1	0	0	1	6	0	6	6/1	51	0	0	↙
4	M	50	Late onset agammaglobulinaemia	5	3	2	10	15	3	18	18/10	260	0	0	N
5	M	25	IgG-IgA deficiency	17	7	10	34	25	15	40	40/34	107	0	76	↙
6	M	43	Acquired agammaglobulinaemia. Isologous BM transplantation	10	3	2	15	7	2	9	9/15	108	8	0	↙↙
Sarcoidosis															
7	M	29		17	10	9	36	9	6	15	15/36	1410	342	135	↙
8	F	36		12	8	5	25	16	5	21	21/25	1400	147	40	↙↙
9	F	53		15	11	7	33	14	9	23	23/33		ND*		↙
10	M	34		0	0	0	0	0	0	0	0/0	2080	345	152	↙↙
11	F	33		13	3	5	21	10	4	14	14/21	1115	168	125	↙
12	M	36		27	5	10	42	15	8	23	23/42	805	153	40	N
13	M	38		12	0	3	15	12	1	13	13/15	1070	375	200	↙
14	M	57		7	4	0	11	2	3	5	5/11	1100	415	105	↙↙
Thymectomy															
15	F	56	Myasthenia gravis	10	3	2	15	9	4	13	13/15	1450	156	186	ND*
16	M	27	Myasthenia gravis	4	9	5	18	10	5	15	15/18	1450	210	135	N
17	F	55	Myasthenia gravis, thymoma	17	5	6	28	13	12	25	25/28	1450	247	160	↙
18	F	41	Parathyroid tumour	10	3	6	19	13	8	21	21/19		ND*		N
19	F	4	Thymus hypertrophy	16	6	4	26	7	15	22	22/26	990	83	53	N
Bone marrow aplasia															
20	F	38	Before BM transplantation	0	0	0	0	37	5	42	42/0	Normal on IEP†			N
			1 month after BM transplantation	4	9	3	16	7	4	11	11/16		ND*		ND*
			5 months after BM transplantation	10	6	4	20	14	10	24	24/20	900	147	185	↙↙

* ND = not done. † IEP = immunoelectrophoresis.

antisera. Predominance of γ chain-bearing lymphocytes was the only relationship between the serum level of immunoglobulins and immunoglobulins on the lymphocytes.

Cell-mediated immunity was not tested in our controls. It had been previously evaluated in eighty-two normal subjects with the following results: 100% had increased incorporation of [^{3}H]thymidine upon stimulation with PHA, 62% responded to SK/SD, 45% to *Candida albicans* and 43% to PPD. Eight (9·7%) did not respond to any of the three antigens. 25·6% of subjects had one test positive, 41·5% two and 23·2% three.

Immunoglobulin deficiency syndromes (Table 2)

All patients had suffered to various extents from recurrent bacterial infections. Four of them (Nos 2, 3, 4 and 6) were treated with γ-globulin when investigated.

Both patients with X-linked agammaglobulinaemia had greatly reduced immunoglobulin-bearing lymphocytes. Cell-mediated immunity was normal in case 1; lymphocytes of patient 2 were stimulated by PHA but not by either of the three antigens (not necessarily abnormal in a 3-year-old boy). Patients 3 and 4 with primary agammaglobulinaemia of late onset were similar. Patient 5 with no detectable serum IgA had normal IgA-bearing lymphocytes. All these five patients had more cells reacting with anti-L chain than with anti-H chain antisera. Patient 6 had proven acquired agammaglobulinaemia; he had received a bone marrow transplantation from a normal monozygous twin in 1966 (Cruchaud, Laperrouza & Mégevand, 1968). His cell-mediated immunity was strongly depressed; L chains were detected on the surface of fewer of his lymphocytes than H chains.

Thirty and 20% respectively of cells from patients 2 and 3 were stained with IgG from normal rabbit serum. Morphological pattern on smears and incubation with polystyrene particles suggested that these cells belonged to the phagocytic mononuclears. Increase of peripheral blood monocytes is known to occur in agammaglobulinaemic patients (M. D. Cooper, personal communication). These values were subtracted from the data presented in the Table.

Sarcoidosis (Table 2)

Patients 8, 9 and 12 had percentages of lymphocytes bearing L and H chains within the normal range. In patient 7, the percentage of cells with H chains was normal but those with L chains were reduced. Patients 4, 5, 7 and 8 had a decreased percentage of both types of chains. In seven cases, more cells had H chains than L chains. Patient 4 had no immunoglobulin-bearing cells other than the 24% of cells identified as monocytes by morphological and functional criteria. μ and λ chains were detected less often than any other chain. In all patients but one, there was a moderate to severe decrease of cell-mediated immunity.

Thymectomy (Table 2)

Lymphocytes bearing H chains were detected with a decreased frequency in three cases and L chains in two. Patient 5 had reversed $\kappa:\lambda$ ratio (also seen in two healthy subjects). Cell-mediated immunity was decreased in one patient only.

Bone marrow aplasia (Table 2)

In patient 20 with bone marrow aplasia due to chloramphenicol, haemoglobin was 6·4 g%, reticulocyte count 0·8%, WBC count 2050/mm^3 (83% lymphocytes and 6% monocytes), and platelet count 10,000/mm^3. Serum immunoglobulins were normal on immunoelectro-

phoresis. PHA and mixed lymphocyte responses were normal.* None of the lymphocytes reacted with any of the anti-H chains antisera; however, the percentage of L chain-bearing cells was normal. Five months following transplantation of bone marrow obtained from a donor with only two identical HL-A antigens, H chains were detected on lymphocytes and the number of cells with L chains was low but in the normal range. Lymphocyte response to PHA and the three antigens was markedly impaired and it was at the lower limit of normal in mixed lymphocyte culture. The patient was still thrombocytopenic, and it was not possible to trace any donor cell in her blood.

Reproducibility of results

Three patients with immunoglobulin deficiency syndrome (Table 2, cases 2, 3 and 6) were reinvestigated after 4–5 months, and one 10-day-old boy (not included in this study) was investigated twice in a 3-day period. Nineteen out of twenty values for surface immunoglobulins showed very good reproducibility; only one value was clearly different from the original one. Investigation was also repeated in our patient with bone marrow aplasia before transplantation, with reproducible results.

DISCUSSION

Using indirect immunofluorescence we found, as other authors, about 30% of fluorescent lymphocytes in healthy subjects with a predominance of IgG-bearing cells (Papamichael *et al.*, 1971; Cooper *et al.*, 1971; Grey *et al.*, 1971). This is comparable to results obtained with a different method by Jondal, Holm & Wigzell (1972); these authors also found that approximately 30% of peripheral blood lymphocytes possessed both surface immunoglobulins and complement binding sites and were unable to form non-immune rosettes, three characteristics which designated them as bone marrow-dependent cells.

L chains were detected more frequently than H chains in nine of thirteen normal subjects. This excess might represent cells carrying IgD and IgE that were not sought for in the present work but which may account for up to 6% of peripheral blood lymphocytes (Aisenberg & Bloch, 1972; van Boxel *et al.*, 1972; Piessens *et al.*, 1973).

The results of patients with immunoglobulin deficiencies confirm previous reports (Cooper & Lawton, 1971, 1972; Grey *et al.*, 1971; Siegal *et al.*, 1971): immunoglobulin-bearing cells were few in X-linked agammaglobulinaemia, and there were fewer H chains than L chains. Two patients with immunoglobulin deficiency had lymphocytes carrying immunoglobulins in the absence of the corresponding immunoglobulins in serum.

Increased immunoglobulin-bearing cells might be expected in sarcoidosis and following thymectomy, and have been reported in sarcoidosis (Holborow, 1972). However, we found normal numbers in five of thirteen patients, they were decreased in five and not detected in one; in two patients either L or H chains were detected with a lower frequency. This may result from three possibilities: (1) B-lymphocytes are increased but a number of them do not synthesize immunoglobulins; (2) B-lymphocytes are increased but some of them carry immunoglobulins (for instance intramembranous) that cannot be detected by our technique; (3) T- and B-lymphocytes are decreased and there is a third population of lymphocytes that cannot be identified as either B or T.

* These tests were performed by Dr Michel Jeannet, Transplantation Immunology Unit, Division of Immunology and Allergy, Department of Medicine, Geneva.

The absence in serum of immunoglobulins which were, nevertheless, present on lymphocytes in some of our patients with immunoglobulin deficiency has already been reported, and was interpreted as a defect of maturation from the stage of lymphocyte to that of plasma cell (Grey *et al.*, 1971; Siegal *et al.*, 1971; Cooper & Lawton, 1972). This suggests that circulating B-lymphocytes may not be representative of immunoglobulin-synthesizing cells in lymphoid tissues, and was confirmed by the observation made in three sarcoidosis patients of normal or elevated serum IgG, IgA or IgM with absence of lymphocytes carrying the corresponding immunoglobulins. In the same perspective, our patient with bone marrow aplasia had no H chain-bearing lymphocytes contrasting with a normal percentage of L chain-bearing cells and normal serum immunoglobulins.

After thymectomy, and in sarcoidosis with defective cell-mediated responses, L chains were uniformly detected with a lower frequency than H chains, contrasting with the usual slight excess of L chain-bearing cells in healthy subjects. The reverse was found in our patient with aplastic anaemia, in whom no H chains were detected on lymphocytes whereas L chains were found with a normal frequency.

Though surface immunoglobulins are considered as characteristic for B-lymphocytes, several studies suggest that thymus-dependent cells in humans and mice also have membrane immunoglobulins of which L chains are more easily detected than H chains (Greaves, Torrigiani & Roitt, 1969; Greaves, 1970; Mason & Warner, 1970; Lesley, Kettman & Dutton, 1971; Bankhurst, Warner & Sprent, 1971; Nossal *et al.*, 1972). Perhaps the indirect fluorescence method would detect L chains on a limited population of T-lymphocytes that may have a particularly high density of surface immunoglobulins.

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