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Inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC) as a means of detection of immune complexes in the sera of patients with thyroid disorders and bronchogenic carcinoma

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SUMMARY

Circulating immune complexes in the sera of patients with thyroid disorders or bronchogenic carcinoma were investigated using an assay system based on the inhibition of the ADCC activity of rat spleen cells. Increased inhibition, as compared with that of the sera of age and sex matched controls, was found in patients with Hashimoto thyroiditis, primary hypothyroidism and bronchogenic carcinoma. The degree of inhibition in the first two groups was markedly increased compared with control sera of the same IgG content. However, the results in the lung cancer group were slightly but not significantly greater than in controls with the same level of IgG.

Increased IgG levels were found in patients with Hashimoto thyroiditis and thyrotoxicosis, and also in patients with bronchogenic carcinoma who had mediastinal gland involvement.

The lower level of sensitivity of the assay system was approximately 600 ng added aggregated IgG, corresponding to a concentration of 6 μg/ml in the sample assayed. It is possible that circulating immune complexes may exist in lung cancer, but at a level below that of the present assay system.

INTRODUCTION

Circulating immune complexes have been implicated as participating in the development of both autoimmune disease, such as systemic lupus erythematosus (Tan *et al.*, 1966; Nydegger *et al.*, 1974; Onyewotu, Holborow & Johnson, 1974; Cowdery, Treadwell & Fritz, 1975), rheumatoid arthritis (Luthra *et al.*, 1975) and glomerulonephritis (Michael *et al.*, 1966; Theofilopoulos *et al.*, 1974), and malignancy (reviewed by Hellström & Hellström, 1974).

One of the methods for the detection of such complexes is based on the inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC), as a consequence of competition between the soluble complex under investigation and the antibody-coated target cell for the Fc receptor on the effector cell (Jewell & MacLennan, 1973).

Preliminary work in this laboratory, using human lymphocytes as effector cells in an assay of this type, demonstrated significantly increased inhibition of ADCC by the sera of patients with Hashimoto thyroiditis, as compared with age and sex matched control sera (Calder & Irvine, 1975).

Using human cells, the degree of target-cell lysis was found to vary considerably, depending on the donor used for preparation of lymphocytes. In order to minimize the variability of the system, spleen cells from an inbred strain of rats have been used as effector cells in the experiments reported in the present paper.

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MATERIALS AND METHODS

Reagents. Eagle's medium (1× strength, without glutamine), foetal calf serum (FCS) and L-glutamine (200 mM) were obtained from Gibco: Bio-cult Ltd, Paisley. The foetal calf serum was heat-inactivated, and the Eagle's medium supplemented with 10% FCS, 2 mM glutamine and antibiotics before use.

Antiserum to chicken red blood cells (CRBC). The antiserum was prepared by repeated intramuscular injection of 1 ml of a 10% suspension of CRBC in Freund's complete adjuvant (1:1). The rabbits were bled 10 days after each injection and the sera tested for potency in the ADCC assay. The sera obtained after two injections were found to be suitable for use in the assay. The animals were boosted 5 months later to obtain more antiserum. All antisera were heat-inactivated at 56°C for 30 min prior to use.

Serum samples. Serum samples were obtained from patients with Hashimoto thyroiditis, primary hypothyroidism, untreated and treated thyrotoxicosis, and bronchogenic carcinoma. Fourteen out of twenty-three of this last group had undergone surgery; some of these had subsequently been treated with BCG. Control sera were obtained from age- and sex-matched hospital patients with illnesses not considered to be associated with circulating immune complexes and from healthy persons including hospital personnel. The sera were stored in 250 μl aliquots at −20°C, and heat-inactivated as described above prior to use. Each sample underwent only one freeze–thawing cycle.

Preparation of effector cells. Preliminary studies in this laboratory have shown considerable variation in ADCC between spleen cells from different inbred strains of rat. One of the strains, namely Liverpool Hooded, whose spleen cells were among the more effective, was chosen for this work. 9–15-week-old male rats were rapidly killed by asphyxiation in a carbon dioxide atmosphere. The spleen was removed, cleaned, and gently hand-homogenized in a few millilitres of Eagle's medium. The cell suspension was decanted, centrifuged at 400 *g* for 5 min, resuspended in 10 ml Eagle's medium+10% FCS, and glass-incubated at 37°C for 2 hr. The non-adherent cells were then removed, spun down and resuspended in 20 ml Eagle's medium+10% FCS. The leucocytes were counted, and the suspension diluted to the required concentration.

The viability of the cells, as assessed by staining with Trypan Blue, was approximately 90%.

Preparation of target cells. Peripheral blood was obtained from 4–12-week-old chickens and centrifuged at 200 *g* for 10 min. The serum and buffy coat were discarded, and the cells washed twice in Eagle's medium and once in phosphate-buffered saline (PBS).

50 μl of an 8% suspension of the chicken red blood cells (CRBC) were incubated with 20 μl (200 μCi; 100–400 mCi/mg) ^{51}Cr-labelled sodium chromate (Radiochemicals Ltd, Amersham) at 37°C for 2 hr. The cells were then washed three times, made to 2 ml in Eagle's medium+10% FCS, counted, and diluted to 2×10^6/ml.

Inhibition of ADCC. The assay used was a modification of that of MacLennan (1972). Incubations were set up in triplicate in LP3 tubes (Luckham Ltd, Sussex), and the mean of the results taken.

Two sets of tubes containing 200 μl of the spleen cell suspension and 100 μl of Eagle's medium+10% FCS were prepared. For each dilution of serum tested, two sets of tubes containing 200 μl of spleen cells and 100 μl of serum dilution were set up. Incubation was carried out at 37°C for 2 hr in an air–5% CO_2 atmosphere. To one set of tubes was added 100 μl of appropriately diluted anti-CRBC antiserum, and, to the other, 100 μl of Eagle's medium+10% FCS. 20 μl of 2×10^6/ml CRBC were then added to each tube, and incubation carried out at 37°C for 16 hr in an air–5% CO_2 atmosphere. Complete lysis of the target cells was effected by 400 μl distilled water.

The tubes were then centrifuged at 200 *g* for 10 min, 200 μl of supernatant removed, and the radioactivity estimated on an automatic gamma-counter.

In the few cases where release in the presence of serum and absence of antibody exceeded that in the absence of serum and absence of antibody, indicating natural anti-CRBC antibodies, the sample and control sera were absorbed with an equal volume of packed CRBC for 1 hr at 4°C, and reassayed.

The percentage inhibition was expressed as:

$$\frac{\%\text{ release in absence of serum sample} - \%\text{ release in presence of serum sample}}{\%\text{ release in absence of serum sample}} \times 100.$$

Label release in the absence of antibody was less than 5%.

Results were analysed for statistical significance by Student's *t*-test.

Serum IgG estimations. Serum concentrations of IgG were determined, using commercial radial immunodiffusion plates (Searle Diagnostic Ltd).

Aggregation of IgG. Purified human IgG was kindly provided by the Blood Transfusion Service, Edinburgh. The concentration was adjusted to 40 mg/ml in PBS, and the solution heated at 63°C for 15 min. The suspension was used without further treatment.

RESULTS

ADCC mediated by rat spleen cells. Considerable ADCC activity was mediated by the rat spleen cells. The degree of lysis of the target cells effected by different ratios of leucocytes to target cells is shown in Fig. 1. With increasing numbers of effector cells the degree of cytolysis increases, reaches a maximum, and then declines. This phenomenon probably results either from utilization of all available nutrients

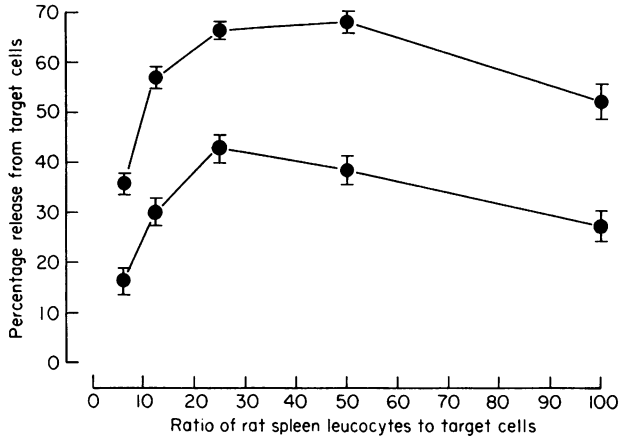

FIG. 1. Lysis of target cells by rat spleen cells. Results shown are the mean and standard error of experiments with six rats. Lower line: antibody concentration of 2×10^{-3}; upper line: antibody concentration of 4×10^{-3}.

or from adverse pH conditions generated due to the large number of cells present. For further studies, a ratio of leucocytes/targets lower than that giving maximal release was used.

Sensitivity of the assay to aggregated IgG. The degree of inhibition of the assay at different concentrations of aggregated IgG is shown in Fig. 2. The limit of detection at a ratio of 20:1 leucocytes/targets and an antibody concentration of 2×10^{-3} appears to be of the order of 6 μg/ml of added aggregated IgG, which corresponds to a final concentration of 1·5 μg/ml in the culture tube.

The limits of detection at a ratio of 50:1 leucocytes/targets and an antibody concentration of 4×10^{-3} was approximately 25 μg/ml of added aggregated IgG, or a final concentration of 6·25 μg/ml.

Results of assays using sera from patients. (a) *Thyroid disorders.* These studies were performed at a ratio of 50:1 leucocytes/targets and an antiserum concentration of 4×10^{-3}.

Sera from twenty-five patients with Hashimoto thyroiditis, twenty-nine patients with primary hypothyroidism, twenty-six patients with untreated thyrotoxicosis and twenty-seven patients with treated thyrotoxicosis were tested for inhibitory activity; the results are shown in Table 1.

Comparing the same dilution of test and control sera, significantly increased inhibitions were obtained with all dilutions of the sera of patients with Hashimoto thyroiditis or hypothyroidism, as compared with age and sex matched controls, whereas no significant difference was found with the thyrotoxic group, except at the two lowest dilutions of sera.

To produce similar degrees of inhibition, control sera had to be used at concentrations more than twice that of Hashimoto thyroiditis or hypothyroid sera.

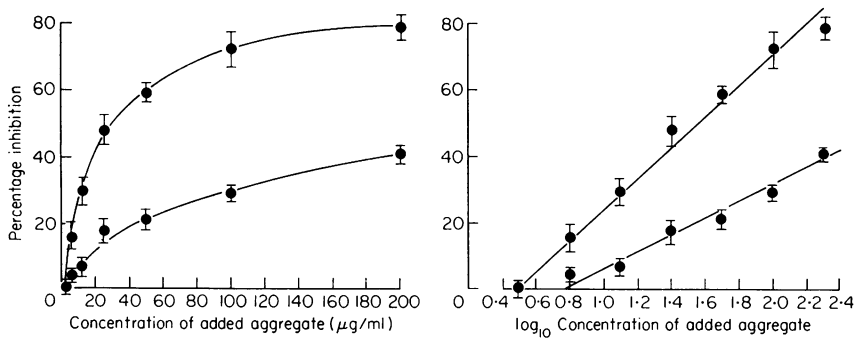

FIG. 2. Inhibition of cytolysis by aggregated IgG. Results shown are the mean and standard error for five experiments. Lower line: ratio of spleen cells to target cells of 50:1, antibody concentration 4×10^{-3}; upper line: ratio of spleen cells to target cells of 20:1, antibody concentration 2×10^{-3}.

TABLE 1. Percentage inhibition (mean ± SE) of ADCC by sera from patients with thyroid disorders

Sample	No. of samples	Dilution of serum							
		10		20		40		80	
Hashimoto thyroiditis	25	35·4 ± 3·54	$P < 0{\cdot}1\%$	24·0 ± 2·84	$P < 0{\cdot}1\%$	15·0 ± 1·92	$P < 0{\cdot}1\%$	11·7 ± 1·70	$P < 0{\cdot}1\%$
Controls		20·5 ± 1·99		10·2 ± 1·42		5·40 ± 0·87		1·56 ± 0·48	
Primary hypothyroidism	29	29·6 ± 3·09	$P < 0{\cdot}1\%$	19·8 ± 2·23	$P < 0{\cdot}1\%$	11·5 ± 1·95	$P < 1\%$	7·03 ± 1·58	$P < 2{\cdot}5\%$
Controls		16·5 ± 1·95		10·3 ± 1·45		5·90 ± 0·91		3·00 ± 0·78	
Untreated thyrotoxicosis	26	24·0 ± 2·12	$P = 5\%$	15·5 ± 2·00	$P < 5\%$	7·16 ± 1·31	$P > 70\%$	3·08 ± 1·01	$P > 70\%$
Controls		17·0 ± 2·63		9·95 ± 2·04		6·50 ± 1·51		3·16 ± 0·97	
Treated thyrotoxicosis	27	26·4 ± 3·31	$P = 5\%$	17·2 ± 2·46	$P = 5\%$	9·00 ± 1·58	$P > 10\%$	4·50 ± 0·98	$P > 70\%$
Controls		19·3 ± 1·92		10·8 ± 1·50		5·80 ± 1·23		3·18 ± 0·84	

TABLE 2. Percentage inhibition (mean ± s.e.) of ADCC by sera of patients with bronchogenic carcinoma

Samples	No. of samples	Dilution of serum							
		10		20		40		80	
Bronchogenic carcinoma	23	61·7 ± 2·40	$P < 5\%$	48·5 ± 2·69	$P < 2{\cdot}5\%$	34·5 ± 2·73	$P > 5\%$	24·6 ± 2·46	$P > 5\%$
Controls		54·0 ± 2·71		40·5 ± 1·94		28·1 ± 1·92		19·7 ± 1·42	
Mediastinal gland involvement	12	63·5 ± 4·16	$P < 5\%$	53·0 ± 4·04	$P < 5\%$	38·5 ± 4·45	$P > 5\%$	29·0 ± 3·58	$P < 2{\cdot}5\%$
Controls		51·0 ± 3·38		39·6 ± 2·83		28·7 ± 3·06		19·4 ± 1·91	
No mediastinal gland involvement	11	59·7 ± 2·23	$P > 90\%$	43·6 ± 3·05	$P > 90\%$	30·1 ± 2·56	$P = 90\%$	19·9 ± 2·83	$P > 90\%$
Controls		57·1 ± 4·25		41·5 ± 2·74		27·5 ± 2·41		20·0 ± 2·23	
No surgery	9	62·4 ± 4·03		47·9 ± 5·28		35·5 ± 5·25		25·5 ± 5·52	
Controls	9	54·8 ± 4·15	$P > 20\%$	41·6 ± 2·52	$P > 30\%$	30·6 ± 1·87	$P > 40\%$	19·8 ± 2·22	$P > 30\%$
Surgery	14	61·2 ± 3·08	$P > 80\%$	49·0 ± 3·02	$P > 80\%$	33·8 ± 3·11	$P > 70\%$	24·1 ± 2·17	$P > 70\%$

TABLE 3. Serum IgG levels (mean ± s.e.) of patients with thyroid disorders

Sample	No. of samples	Serum IgG level (mg/100 ml)	*P*
Hashimoto thyroiditis	25	1642 ± 84·4	< 0·1%
Controls		1189 ± 53·6	
Primary hypothyroidism	29	1256 ± 44·9	> 10%
Controls		1133 ± 60·7	
Untreated thyrotoxicosis	26	1329 ± 61·8	< 0·5%
Controls		1088 ± 43·7	
Treated thyrotoxicosis	27	1216 ± 47·2	> 10%
Controls		1105 ± 61·6	

(b) *Bronchogenic carcinoma.* As the levels of immune complex in cancer patients might be expected to be fairly low, the more sensitive assay conditions of a 20:1 ratio of leucocytes/targets and antibody concentrations of 2×10^{-3} were used.

The degree of inhibition produced by sera from twenty-three lung cancer patients is shown in Table 2. Statistically significant increases in inhibition, as compared with age and sex matched control sera, were obtained at the two lowest dilutions of the sera for the whole group, and at three dilutions for the subgroup of patients with mediastinal gland involvement. Serum from this subgroup was as inhibitory as control sera at half the concentration of the control sera, whereas no difference in inhibition was obtained between control sera and that of patients without glandular involvement.

No statistically significant difference was obtained between the group of patients who had not been subjected to surgery and either the appropriate controls or the group which had undergone surgery.

Determination of serum IgG levels. Increased inhibition of ADCC by patients' sera could result from a number of factors including increased levels of aggregated IgG. To investigate the latter possibility, determinations of IgG levels were carried out on the same sera as used in the inhibition assay.

(a) *Thyroid disorders.* The results for this group are shown in Table 3. Statistically highly significant elevations of IgG levels were found in two groups, the greatest elevation being in the Hashimoto thyroiditis patients, a smaller increase being found in the untreated thyrotoxic patients.

(b) *Bronchogenic carcinoma.* The results for this group are shown in Table 4. Statistically highly significant elevations of IgG were found in the group as a whole. However, this increase appeared to be restricted to the subgroup with glandular involvement.

TABLE 4. Serum IgG levels (mean ± SE) in patients with bronchogenic carcinoma

Sample	No. of samples	Serum IgG levels (mg/100 ml)	*P*
Bronchogenic carcinoma	23	1621 ± 93·0	< 1%
Controls		1298 ± 58·2	
Glandular involvement	12	1749 ± 140	< 0·5%
Controls		1242 ± 72·4	
No glandular involvement	11	1481 ± 112	< 40%
Controls		1360 ± 87·4	
BCG-treated patients	12	1527 ± 58·4	< 60%
Non-BCG-treated	14	1605 ± 159	

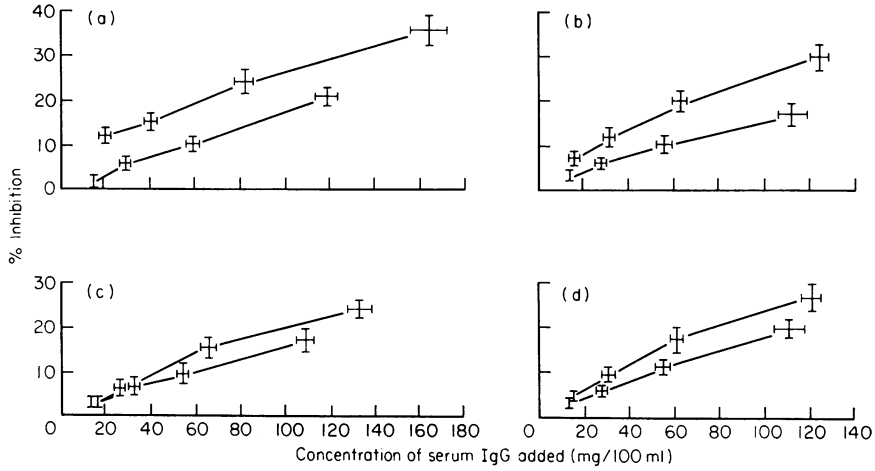

FIG. 3. Comparison of inhibition of ADCC with serum IgG levels—thyroid disorders. Results shown are the mean and standard error. (a) Hashimoto thyroiditis; (b) primary hypothyroidism; (c) untreated thyrotoxicosis; (d) treated thyrotoxicosis. Upper line, patients' sera; lower line, control sera.

Correlation of inhibition with the IgG content of the test serum. In order to determine if the inhibition observed was merely resulting from aggregation of the IgG in the sera used, the percentage inhibition observed was compared with the concentration of IgG in the samples assayed.

(a) *Thyroid disorders.* The results are shown in Fig. 3. It is apparent that, at the same level of IgG, sera of patients with Hashimoto thyroiditis or hypothyroidism are markedly more inhibitory than those of controls. However, the difference between the sera of thyrotoxicosis patients and controls is only slight.

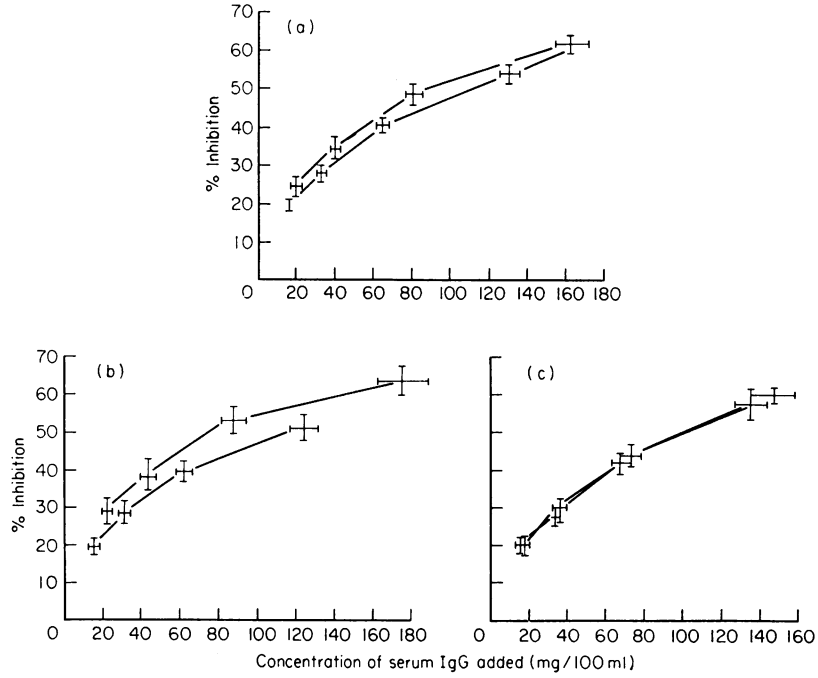

FIG. 4. Comparison of inhibition of ADCC with serum IgG levels—bronchogenic carcinoma. Results shown are the mean and standard error. (a) Total bronchogenic carcinomas; (b) with glandular involvement; (c) no glandular involvement. Upper line, patients' sera; lower line, control sera.

(b) *Bronchogenic carcinoma.* The results are shown in Fig. 4. No statistically significant difference is observed between the total group or either subgroup and the controls.

However, there is some indication that the group with mediastinal gland involvement may show a greater degree of inhibition compared with control sera at the same IgG level.

DISCUSSION

Using the assay system described above, it was possible to demonstrate marked inhibition of ADCC by the sera of patients with Hashimoto thyroiditis and primary hypothyroidism and less marked inhibition with the thyrotoxic group. Inhibition of ADCC could be caused by several factors, such as immune complexes or aggregated IgG, and possibly by other agents, including factors cytotoxic to lymphoid cells or proteolytic enzymes capable of modifying the response, e.g. by digestion of the anti-CRBC antibodies. Evidence in favour of immune complex in these patients is accumulating. Firstly, Kalderon, Bogaars & Diamond (1973) have demonstrated the presence of electron-dense deposits in the follicular basement membrane of the thyroid gland of eight patients with Hashimoto thyroiditis. Secondly, the results of Calder *et al.* (1973a, 1974), Calder, McLeman & Irvine (1973b) and Calder & Irvine (1975), using complement fixation and arming of K cells, are also suggestive of the presence of circulating immune complexes in these patients. Thirdly, the present study demonstrates the presence of material capable of inhibiting ADCC; this is unlikely to be aggregated IgG, as the degree of inhibition does not correlate solely with the serum IgG content. Finally, our preliminary characterization data suggest that the inhibitory factor, or factors, involved is of a size compatible with its being an immune complex.

Elevated inhibition of ADCC was also observed in the sera of patients with bronchogenic carcinoma. However, in contrast with the previous group, the inhibition correlated much more closely with the IgG content of the sera. The subgroup of patients with mediastinal gland involvement showed slightly elevated levels of inhibitory material, but more samples would be required to be examined to determine if this was a significant effect. It appears, therefore, that if circulating immune complexes exist in this disease, such complexes must be present at levels lower than the detection limits of this particular assay. The lower level of sensitivity of the system is approximately 60 μg/ml for aggregated IgG in serum; therefore, if immune complexes inhibit to the same extent as aggregated IgG, the serum levels of the postulated complex must be below this value. It is probable, however, that complexes of different ratios of antigen/antibody may have different affinities for the lymphoid cell Fc receptor, and thereby inhibit to different extents.

By a similar argument, the levels of complex in patients with Hashimoto thyroiditis and hypothyroidism should be present in excess of 250 μg/ml. It might be readily envisaged that circulating immune complexes at such a high concentration could profoundly affect the immune response.

The present studies demonstrate that this particular assay has certain limitations, the most serious being the requirement for heat-inactivation. As can be seen from the data, control sera show considerable inhibitory activity, especially as the sensitivity of the assay is increased. This could, *à priori*, be ascribed to aggregation of IgG induced by the heat-treatment. Furthermore, when heated at 56°C for 30 min at a concentration of 12 mg/ml, pure IgG inhibits the assay to the same extent as control sera, the proportion of the IgG precipitable by 0·8 M sodium sulphate is increased, suggesting aggregation (Christian, 1960); much of the inhibitory material is also precipitated, and, finally, we have observed that heat-inactivated plasma is markedly more effective in releasing radioactive serotonin from human platelets than is non-heat-inactivated plasma. However, aggregation caused by heat inactivation does not alone account for the inhibitory activity of control sera, as decomplementation of the sera by more gentle methods, such as treatment with ammonia, 0·1 M mercaptoethanol or zymosan, or the use of untreated mouse serum causes a similar degree of inhibition. Furthermore, natural antibodies to rat spleen cells do not seem to be responsible as autochthonous rat serum is also inhibitory.

The degree of inhibition produced by control sera is similar to that produced by 400–640 μg/ml of aggregated IgG.

Heat-inactivation, therefore, is to be avoided. Such a step is not necessary in the system of Jewell &

MacLennan (1973), using Chang target cells, as the concentration of antiserum can be adjusted to a level which mediates cytolysis by lymphoid cells but not by complement. Chicken erythrocytes, however, are readily lysed by human serum in the complete absence of added antibody, even after absorption. Similar results are obtained with rabbit serum and chicken serum. The effect cannot be ascribed to natural antibody as chicken serum also lyses autochthonous erythrocytes. The effect is observed in rabbit sera deficient in C6, suggesting either that terminal complement components are not required (C6 may possibly be present on the erythrocyte), or that the effect is not mediated by complement. However, complement is implicated, as the agent involved is destroyed completely by heat-inactivation or treatment with ammonia, is depleted by treatment with zymosan, and correlates with the complement content of the serum. Target-cell bound complement participating in antibody-directed cell-mediated cytotoxicity, as reported by Perlmann *et al.* (1969), O'Neill, Mackler & Wyde (1975) and Lustig & Bianco (1976) and suggested by the results of Eden, Bianco & Nussenzweig (1973) and Scornik & Drewinko (1975), does not appear to be the cause as the effect is also observed in the presence of cytochalasin B, an agent which effectively blocks cell-mediated cytotoxicity (e.g. Gelfand, Morris & Resch, 1975), and in the absence of lymphoid cells. Complement activation by chicken erythrocytes, similar to that reported for human and rabbit platelets (Zimmerman & Kolb, 1976), mouse spleen cells (Pepys, 1974), tumour cells (Okada & Baba, 1974) and rabbit cells (Platts-Mills & Ishizaka, 1974) cannot be excluded.

Elevated IgG levels were detected in the sera of patients with Hashimoto thyroiditis and lung cancer. The results with the Hashimoto patients bear out the early work of Lewis & McCullagh (1944), Skillern *et al.* (1956) and Luxton & Cooke (1956) but conflict somewhat with those of Glynne & Thomson (1972) who, in a study of serum IgG levels in thyroid disorders, found elevated levels only in relapsed Graves' disease patients. Elevated levels in lung cancer patients were reported previously by Hughes (1971), but this author made no further breakdown of the data. Although the number of samples tested is admittedly small, the present evidence suggests that this increase is confined to those patients with mediastinal gland involvement. It is relevant to note that the increased IgG levels in the lung cancer patients is not a result of BCG treatment. As can be seen from Table 4, both BCG and non-BCG treated patients have elevated levels. Furthermore both the group with mediastinal gland involvement and the group without contained similar proportions of BCG-treated patients.

Finally, it is of interest to note that the response, i.e. the inhibition, is not linearly related to the concentration of aggregated IgG (Fig. 2). One explanation is that the binding of one aggregate inhibits the binding of further aggregates. This could be explained by the suggestion of Wiedermann *et al.* (1975) that efficient binding via Fc receptors requires binding by a clump of Fc regions. Binding of a few aggregates could thereby markedly reduce the availability of potential clumping sites. A further possibility is that different cell-types, with Fc receptors of differing affinities, are being inhibited.

Present work involves the isolation and characterization of the inhibitory factors in the patients with autoimmune disease, and in the study of the lung cancer patients, the development of more sensitive assays for immune complexes.

Addendum

Since this paper was submitted, Theofilopoulos, Wilson & Dixon (1976) have reported evidence for circulating immune complexes in the sera of patients with serum hepatitis, SLE, vasculitis, subacute sclerosing panencephalitis, dengue haemorrhagic fever and various malignancies. In the case of lung cancer, in two out of seven patients they observed levels of complex corresponding to 26 μg/ml of aggregated IgG. This low level would be below the level of detection of the above study.

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