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Immunocomplexes and Tumour Growth. Detection of Immunocomplexes in High and Low Malignant Tumour Sublines

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Two different techniques to measure immunocomplexes on biological fluids are presented. The first one is based on the inhibition of the antibody dependent cell cytotoxicity (ADCC). A competition is stablished between immunocomplexes and the rabbit anti chicken antibodies bound to chicken red blood cells for the Fc receptors of K cells. The second technique detects the presence of immunocomplexes by the inhibition of the citotoxicity on a haemolytic system as a result of the consumption of the complement. Both techniques were performed in parallel to compare their sensitivity. The presence of immunocomplexes in the ascites of tumour bearing mice has been demonstrated with two highly related tumours (MCG4 O and MGC4 C) with different immunogenic properties. No immunocomplexes were detected in the serum of the same mice.

Some tumour cells grow on syngeneic animals despite a strong immunoreaction (13). Isoantibodies as well as cytotoxic T lymphocytes have been generated by and directed against tumour specific antigens (8, 20). Recently, tumour antigens have been correlated with foreign transplantation antigens (H-2) and humoral as well as cellular immune response have been induced against them (6, 9, 10, 13, 16-18).

Immunocomplexes (I.C.) are known to induce autoimmune diseases and to stimulate tissue damage in some cases (12). For tumour growth, soluble tumour antigens are believed to create a protection against the attack of specific antibodies and lymphocytes, by blocking antigen receptors (1). According to this hypothesis the presence of immunocomplexes will reflect a failure in rejecting the tumour and thus provide an index of the degree of malignancy.

This paper presents the development of two different techniques to measure I.C. in mice, previously injected with MCG4 O and MCG4 C tumour cells,

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whose antigenicity and malignancy (9, 10. 19, 20), had been priorly characterized. The first technique is based on the blocking of Fc receptors of K cells by immunocomplexes with inhibition of the antibody dependent cell cytotoxicity (ADCC). The second one uses the property of immunocomplexes to activate complement, reducing the capacity to lyse a haemolytic system.

Materials and Methods

Culture medium. MEM (Eagle 10×, Wellcome Research Laboratories) supplemented with 2.5 % sodium bicarbonate (7.5 % Flow Laboratories), 5 % foetal calf serum (Flow Laboratories) inactivated 1 h at 56° C and 1.5 % Hepes buffer (1 M, Gibco Laboratories).

Target cells. Chicken erythrocytes were obtained from the axillary veins with a syringe containing heparin (Leo Laboratories), washed twice with PBS (phosphate buffer saline) and labelled with 50 μCi of ^{51}Cr (sodium chromate) for 1 h at 37° C. Routinely 20×10^6 erythrocytes were labelled for each experiment. After labelling, the erythrocytes were washed twice with PBS and adjusted to a concentration of 1×10^5/ml (for ADCC) and 2×10^6 (for CDL).

Effector cells. Normal mouse lymphoid cells were obtained from spleen of the following congenic recombinant strain of mice: B10.A (4R) (H-2^{b4}), B10. HTT (H-2^{t3}), and C57B1/6 (H-2^b). The different strains of mice were bred in our animal house facilities from breeding pairs obtained from Prof. H. Festenstein (The London Hospital) and Prof. V. Shirrmacher (Deutches Krebsforschungzentrum, Heidelberg). The spleen cells obtained were washed twice with PBS and adjusted to a concentration of 10^7 cells/ml.

Antisera. Rabbit anti chicken antiserum (RACA) was obtained by intravenous injections of chicken erythrocytes (ten injections of 1 ml/kg of 10 % erythrocytes solution on PBS). The xenoantiserum obtained was heated, inactivated, and frozen at —30° C. The titer of the xenoantiserum was 1/75,000.

Rabbit anti mouse gammaglobulin (rabbit anti MGG) was obtained by four subcutaneous injections of 1 mg Ig/ml of PBS in complete Freund adjuvant, followed on the 5th week by one intravenous injection. The rabbit was bled on the 6th week. Antibodies anti mouse Ig were demonstrated by precipitation in agar.

Complement. Fresh rabbit serum has been used as a source of complement. It was kept at —170° C in liquid nitrogen. The complement was titrated on complement dependent cytotoxicity and CH50 was established at 1/180 dilution.

Positive control for immunocomplexes. Several immunocomplexes were prepared by incubating normal mouse serum from B10.HTT, ASW and B10 mice with the xenoantiserum rabbit anti MGG for 30 min at room temperature.

Preparation of samples of serum and ascites fluid from tumour bearing mice. BALB/C mice were intraperitoneally injected with MCG4 O and MCG4 C tumour cells. 3×10^6 tumour cells were injected each time and serum and ascites fluid was obtained one week after inoculation of the tumours. Samples were heat inactivated for 30 min at 56° C. The origin and immunogenic properties of the sublines MCG4 O and MCG4 C have been reported elsewhere (20).

Detection of immunocomplexes by inhibition of the antibody dependent cell cytotoxicity (ADCC). Normal mouse spleen cells at a 10^7/ml concentration were incubated with the control immuno-

complexes or the serum and ascites from tumour bearing animals for 1 h at room temperature. The immunocomplexes, and serum and ascites were prepared at the following dilutions: 1/10, 1/100, 1/1,000, 1/10,000. The final volume of the incubation mixture was 0.4 ml (0.2 ml normal lymphoid cells and 0.2 serum or ascites). On a second step 0.2 ml of a solution (1/25,000) of RACA was added and incubated for 10 min at room temperature. Chicken red blood cells ^{51}Cr labelled (10^5) in 0.2 ml and 10^7 sheep red blood cells in 0.2 ml were added to the mixture. The chicken red blood cells were used as target cells and the sheep red blood cells to decrease the spontaneous ^{51}Cr release. The mixture was incubated for 3 h at 37° C. Finally, the tubes were centrifuged and the supernatant and the pellet were counted independently on a well type gamma counter (Ultrogamma LKB). The percentage of specific ^{51}Cr release was calculated according to the formula:

$$\frac{\text{cpm supernatant of test samples} - \text{cpm supernatant with medium alone}}{\text{cpm supernatant and pellet}} \times 100$$

Results are expressed as % inhibition of ADCC (2).

Detection of immunocomplexes by inhibition of the complement dependent lysis (Complement consumption test CDL). 0.2 ml of different dilutions of serum, ascites and control immunocomplexes were mixed in triplicate tubes with 0.2 ml of rabbit complement at a final dilution 1/180 (CH-50). The tubes were incubated for 30 min at room temperature. Chicken red blood cells ^{51}Cr labelled were incubated with a solution of RACA (1/5,000) for 15 min at room temperature. Finally, both preparations containing 1) the immunocomplexes and the complement, and 2) the haemolytic system, were incubated on a final volume of 0.4 ml for 1 h at 37° C. The tubes were centrifuged and the supernatant counted. The cytotoxic index was calculated according to the formula:

$$\text{CDL}\,\% = \frac{\text{cpm maximum release} - \text{cpm spontaneous release}}{\text{cpm test tubes} - \text{cpm spontaneous release}} \times 100$$

Results are expressed as % inhibition of complement dependent lysis.

Results

Two different cytotoxic techniques have been developed to measure immunocomplexes. The first is dependent on lymphoid cells (ADCC) and the second on complement (CDL). In the first technique the haemolytic system is composed of chicken red cells as target cells, normal mouse spleen cells as effector cells and rabbit anti chicken antibodies (RACA) as xenoantiserum (fig. 1*a*). The degree of lysis of target cells in the presence of RACA and K cells, is measured by the specific ^{51}Cr release to the supernatant (fig. 1*b*). If immunocomplexes are present in the mixture a competition for the Fc receptors of the K cells take place between RACA and immunocomplexes (figure 1*c*). The more immunocomplexes present the greater the inhibition of ADCC becomes.

Figure 2*a* shows an ADCC reaction in relation to the concentration of RACA, in which 40 % of specific ^{51}Cr release is obtained with concentrations of 5×10^{-4}

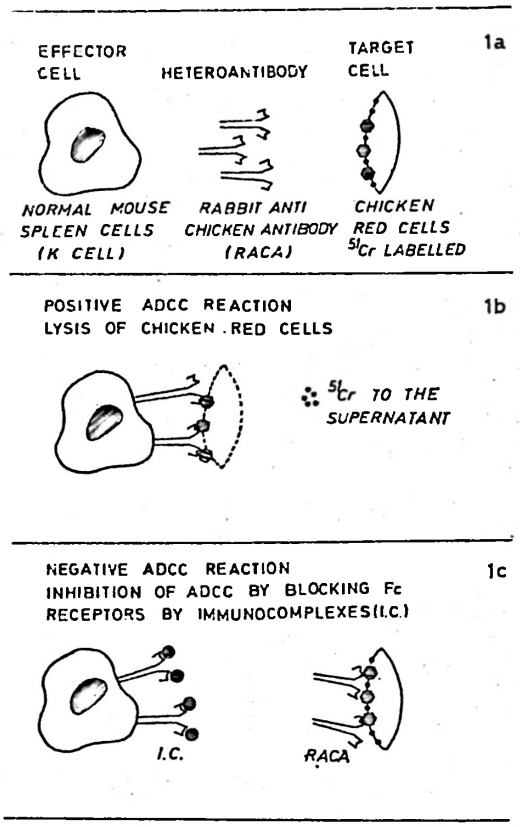

Fig. 1. *Detection of Immunocomplexes by the Inhibition of antibody dependent cell cytotoxicity (ADCC).*
a) Components of the cytotoxicity system. *b*) Normal ADCC reaction measured by the specific ^{51}Cr release to the supernatant. *c*) Inhibition of the ADCC by competition for Fc receptors of K cells by immunocomplexes and the rabbit chicken antibody bound to chicken red cells.

and 10^{-5}. The relationship between target and effector cells was stablished on 1/100. In the routine assay the concentration of RACA was always 25×10^{-4}.

The presence of immunocomplexes in the same samples has been demonstrated by a second technique. It is based on the capacity of immunocomplexes to activated complement. The haemolytic system is composed of chicken red cells as target cells, rabbit anti chicken antibodies and rabbit complement at a concentration that produces 50 % of the lysis (CH50). Tre presence of immunocomplexes will reduce the lysis in the haemolytic system due to the absent of complement. Fig. 2*b* shows the titration curve for complement from 1/5 to 1/500. The CH50 was stablished at a concentration of 1/80.

Experimental immunocomplexes have been used to test the sensitivity and reproducibility of the two different techniques. The complexes mouse Ig and rab-

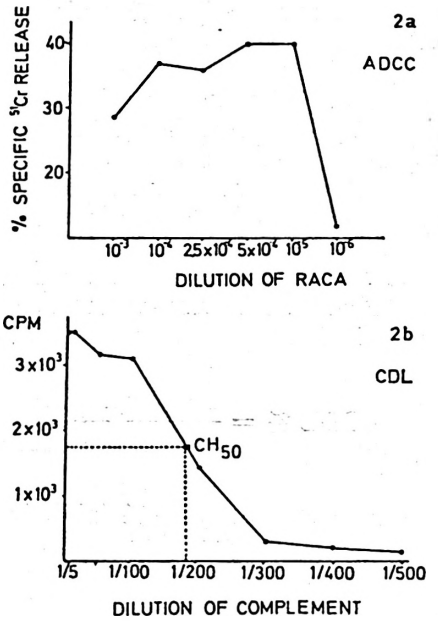

Fig. 2. *Effect of RACA and complement concentration of the ^{51}Cr release.*
a) Percent of specific ^{51}Chromium release obtained in an ADCC reaction. Relation between target and effector cells 1/100. The rabbit anti chicken antibody has been used at various concentrations. *b*) Titration of the rabbit complement on a haemolytic system composed of ^{51}Cr labelled chicken erythrocytes as target cells and rabbit anti chicken antibodies. The CH50 was obtained at a dilution of complement 1/180.

bit anti mouse Ig have been used. Fig. 3 shows that these immunocomplexes inhibit the antibody dependent cell cytotoxicity (ADCC) at all the concentrations used: 87.6 % inhibition at 1/10 concentration, 69.3 % inhibition at 1/100 concentration, 28.6 % at 1/1,000 concentration and 6.4 % for the dilution of IC of 1/10,000. The complement consumption test results show 60 % inhibition for 1/100 concentration and 50 % for 1/1,000.

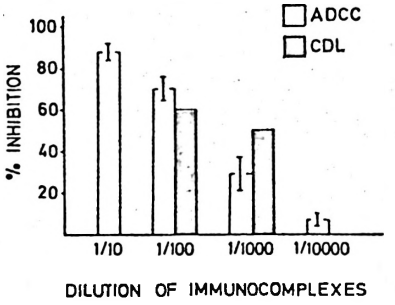

Fig. 3. *Demonstration of the presence of immunocomplexes at different dilutions.* By the inhibition of the ADCC reaction, and by the inhibition of the complement dependent cytotoxicity (CDL).

Both techniques have been applied to measure immunocomplexes in serum and ascites fluid from mice previously injected with MCG4 O and MCG4 C tumour cells. MCG4 is a BALB/c (H-2^d) tumour induced with methylcholantrene on 1977 (7). Recently several sublines have been characterized and named MCG4 O, MCG4 A, MCG4 B, and MCG4 C. While MCG4 O and A are highly immunogenic, MCG4 B and C are not. The different immuno-

Table I. *Inhibition of ADCC and CDL by experimental immunocomplexes (mouse Ig-antI Ig) and serum and ascites fluid of MCG4 O and MCG4 C bearing BALB/c mice.*

	Dilution	% ADCC *	% Inhibition **	% CDL *	% Inhibition **
Cytotoxicity		33.36		64.41	
Experimental I.C.	1/10	3.82	87.85	64.31	0
	1/100	10.22	69.34	25.43	60.64
	1/1,000	23.80	28.63	31.91	50.31
	1/10,000	31.22	6.40	64.53	0
MCG4 O serum	1/10	33.85	0	67.84	0
	1/100	35.07	0	69.15	0
	1/1,000	34.00	0	65.04	0
	1/10,000	33.66	0	65.14	0
MCG4 C serum	1/100	34.03	0	64.85	0
	1/100	33.65	0	65.01	0
	1/1,000	33.91	0	66.43	0
	1/10,000	34.07	0	65.14	0
MCG4 O ascites	1/10	7.17	78.50	64.70	0
	1/100	27.04	18.92	44.14	31.66
	1/1,000	34.17	0	57.02	11.74
	1/10,000	34.04	0	69.01	0
MCG4 C ascites	1/10	13.34	60.00	17.44	73.38
	1/100	23.01	31.00	21.19	67.20
	1/1,000	34.27	0	57.38	11.19
	1/10,000	34.55	0	65.14	0

* Specific ^{51}Cr release: a) In ADCC 33.36 is the mean of several experiments with a ratio target cells/effector cells 1/100 and RACA at 2.5×10^{-5}. b) In CDL 64.41 represent the mean of different experiments with RACA at 5×10^{-3} and rabbit complement at 1/180.

** The percentage of Inhibition has been calculated by taking 33.36 cytotoxicity for ADCC and 64.41 for CDL as 100 %.

genic capacity seems to correlate with the phenotypic H-2 alterations demonstrated with MCG4 sublines (18). MCG4 is herein studied in relation to the production of immunocomplexes when growing in syngeneic BALB/c animals. For this purpose the sublines MCG4 O and MCG4 C, which have so markedly different immunogenic properties have been selected. MCG4 O is highly immunogenic while MCG4 C is not.

The results obtained show that the serum from BALB/c mice injected with MCG4 O or MCG4 C does not contain immunocomplexes as demonstrated by the two different techniques (table I). In contrast, the ascites fluid from the same BALB/c mice gives a positive reaction by inhibiting the ADCC and CDL, showing thereby the presence of I.C.

Discussion

MCG4 O is a highly immunogenic methylcholantrene induced BALB/C tumour that arises from the solid MCG4 as an ascites form (7, 18). It was found to have few H-2^d antigens, to react with antisera against foreign H-2 specificities (H-2.5, H-2K33, H-2D.2) (7, 18) and monoclonal anti H-2^k antibodies (18). Absorption experiments and tumour typing with alloreactive CTL's confirmed these findings (7, 19). MCG4 O was able to induce the production of high titer isoantibodies on syngeneic BALB/C mice. The isoantibodies reacted not only to MCG4 O but to allogeneic tumours and normal allogeneic cells (8). The pattern of reaction of BALB/C anti MCG4 O isoantiserum with normal allogeneic cells revealed a close similarity with that obtained with anti H-2.5 alloantiserum (8, 17, 19, 20).

Since 1977, MCG4 O has been routinely passed i.p. by inoculations of $3\text{-}5 \times 10^6$ tumour cells. In 1979 several sublines of MCG4 were observed to be cleary distinguishable when typing different batches of MCG4 tumour cells. MCG4 sublines have different capacity of growth on syngeneic BALB/C mice. MCG4 A is the most immunogenic, followed by MCG4 O. Both sublines have the property to induce isoantisera (8, 18) and MCG4 A in addition induces cytotoxic T lymphocytes that react specifically with the tumour and also with normal allogeneic cells (17). MCG4 B and MCG4 C in contrast are poorly immunogenic, growing easily in BALB/C mice.

It has been demonstrated that tumour cells can escape the immune response by shedding membrane antigens to the surrounding spaces (3, 15), these tumour antigens can block the recognition sites of antibodies and lymphoid cells forming immunocomplexes that protect the tumour from the immune response (4). It has been also shown that factors present in the sera of tumour bearing host interfere with lymphocyte mediated tumour cells destruction (5, 11). These factors include excess tumour antigen (1, 15) anti tumour antibodies (11) and antigen-antibody complexes (14).

We present here the results obtained in the search for immunocomplexes in the serum and ascites fluid of BALB/C mice inoculated with two sublines of the same tumour MCG4: subline MCG4 O, highly immunogenic, and subline MCG4 C, poorly immunogenic.

Our results show: *a*) that inhibitions of both ADCC and CDL are sensitive techniques to demonstrate the presence of immunocomplexes (2); *b*) that serum from BALB/C mice inoculated intraperitoneally with MCG4 O or MCG4 C does not contain immunocomplexes capable of inhibiting ADCC or CDL, *c*) that the ascites fluid from these mice contains immunocomplexes easily detectable by both assays.

Finally, the malignancy of subline MCG4 C has not been yet correlated to the immunocomplexes present in the se-

rum, while no differences with the immunogenic MCG4 O subline have been found on this respect. Further experiments to analyse other tumours with different immunogenic properties are under way.

Resumen

Se desarrollan dos técnicas para la medida de inmunomplejos en líquidos biológicos. Una de ellas está basada en la inhibición de la citotoxicidad dependiente de anticuerpos y células (ADCC). Los inmunocomplejos a medir compiten junto con los inmunocomplejos presentes en el sistema hemolítico por los receptores Fc de las células K o células efectoras. La otra se basa en la inhibición por la presencia de inmunocomplejos de la citotoxicidad dependiente de complemento como consecuencia de su consumo. Ambas técnicas utilizan un sistema isotópico para cuantificar la inhibición, y se han realizado en paralelo para comparar su sensibilidad. Han sido aplicadas para medir inmunocomplejos producidos experimentalmente en el laboratorio, así como para medir la presencia o no de inmunocomplejos en suero y líquido ascítico de ratones portadores de tumores estrechamente relacionados entre sí por su origen, aunque con grandes diferencias antigénicas. Los resultados obtenidos demuestran que el líquido ascítico producido por ambos tumores contienen inmunocomplejos, mientras que no se detectan en el suero de los mismos animales portadores de estos tumores.

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