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Anti-Tumour Effect of Chlorambucil–Antibody Complexes in a Murine Melanoma System*

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Abstract—*An anti-serum against a transplantable murine melanoma (Harding Passey melanoma; HPM) was raised in rabbits. The IgG fraction of the anti-serum was non-covalently linked to chlorambucil. The chlorambucil–antibody complex was tested in BALB/c mice injected i.p. with HMP cells. The complex had considerably more anti-tumour effect than the unlinked components.*

INTRODUCTION

THE APPLICATION of cytotoxic drugs in the treatment of cancer is limited by the absence of a specific anti-tumour action.

The specific anti-tumour action can be acquired by the use of antibodies with specific affinity for the tumour cells as carriers of cytotoxic drugs. Various drugs have been studied for this purpose, e.g., radionuclides [1, 2], methotrexate [3, 4], anti-diphtheria toxin [5] and, in particular, chlorambucil [6–14].

Regression of all metastatic nodules has been described for a patient with dissiminated melanoma after treatment with chlorambucil linked to goat antibody directed against the tumour [6]. However, the usefulness of the method of complexing chlorambucil and immunoglobulins is still questionable [15–18] despite the positive reports. For instance, it has been shown that a mixture of the drug and antibody or successive injection of chlorambucil and antibody can also be effective in the *in vivo* situation [8, 11, 19, 20]. *In vitro* a mixture of chlorambucil and the specific antibodies can also kill the cultured tumour cells [21, 22].

In this report we describe experiments in which BALB/c mice injected i.p. with Harding Passey melanoma (HPM) cells were treated with a complex of chlorambucil physically linked to immunoglobulin, chlorambucil, immunoglobulin, a mixture of the two, or with successive injections of the drug and the immunoglobulin.

MATERIALS AND METHODS

Mice

Mice were obtained from the Centraal Proefdieren Bedrijf TNO, Zeist, The Netherlands.

Inbred DBA/2 and BALB/c, male and female mice were used at the age of 8–12 weeks.

BALB/c mice were used as a source of Harding Passey melanoma cells and as test mice.

DBA/2 mice were used as a source of SL2 lymphoma cells.

Tumours

The Harding Passey melanoma (HPM) was found on the left ear of a brown stock mouse in the laboratory of R. A. Passey of the Leeds University Medical School (Leeds, England, 1925). More data about the HPM have been summarized by Stewart *et al.* [23]. The tumour sometimes kills the recipient after a

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List of abbreviations: Chl. = Chlorambucil; EDTA = Ethylene diamine tetra acetic acid; FBS = Fetal bovine serum; FCA = Freund's complete adjuvant; GAR-FITC = Goat-anti-rabbit-fluorescein-isothiocyanate; HMP = Harding passey melanoma; i.m. = Intramuscularly; i.p. = Intraperitoneally; PBS = Phosphate buffered saline; s.c. = Subcutaneously.

considerably longer period than usual. This is in agreement with the observation of Sugiura [24] that tumour transplants did not take in 3% of 1500 recipient mice. HPM can be transplanted in BALB/c mice and grows i.p. as well as s.c. as a solid tumour. The tumour is maintained by a weekly i.p. passage of 10^7 cells.

HPM suspensions were prepared by the following method: The tumour was cut and pressed through a gauze. The cells were suspended in Fischer's medium [25]. Clumps of cells were allowed to settle. The supernatant containing free floating cells was centrifuged. The cells were suspended in Fischer's medium and used for the *in vivo* experiments.

For the *in vitro* experiments the free floating cells were cultured in 50 ml Falcon-culture flasks with 10 ml Fischer's medium supplemented with 10% fetal bovine serum (FBS) at 37°C for 2 days. After 2 days the monolayer was washed with medium and the tumour cells were detached from the bottom by incubation with 0.54 mM EDTA at 37°C for 10 min. The cells were washed twice with medium before use in cytotoxicity tests and/or immunofluorescence tests.

SL2 Lymphoma

The SL2 lymphoma is syngeneic to DBA/2 mice and arose spontaneously as an ascitic tumour (Sutton, England, 1969). The tumour grows i.p. as an ascitic tumour and is maintained by weekly i.p. passage of 10^6 cells.

Antiserum

Anti-HPM serum was raised in rabbits. The rabbits received 2 intramuscular (i.m.) injections of 10^7 tumour cells suspended in 1 ml of Fischer's medium, mixed with an equal volume of Freund's complete adjuvant (FCA) with an interval of 2 weeks, followed by an i.m. injection of 10^7 tumour cells without FCA 1 and 2 weeks later.

The serum was collected 7 days after the last immunization and decomplemented at 56°C for 30 min. Control serum was obtained from non-immunized rabbits. Both sera were absorbed twice with equal volumes (v/v) of BALB/c mouse tissue homogenates of liver, heart, spleen and kidney at 4–10°C for 30 min.

Isolation of the globulin fraction

The globulin fractions of the immune and the control sera were isolated by 3 successive precipitations with a $(NH_4)_2SO_4$ solution (final concentration 33.3%). This was followed by dialysis against 0.01 M phosphate buffered saline (PBS: pH 7.2) at 5°C for 24 hr. Globulins were freeze-dried and stored at −20°C. The globulins were reconstituted to PBS before use.

Immunoelectrophoresis [26] of the isolated globulins showed that the globulin fraction mainly consisted of IgG. The fraction will further be denoted as IgG.

Physical linkage of chlorambucil to immunoglobulin

Chlorambucil was linked to the isolated immunoglobulin fractions of the sera by a procedure described by Blakeslee and Kennedy [27]. Chlorambucil was dissolved in 0.06 N NaOH and brought to a concentration of 12.5 mg/ml with PBS (pH 7.2). The chlorambucil solution was added to an equal volume of immunoglobulins dissolved in PBS. The protein concentration in the reaction mixture was 8.25 mg/ml and the protein to chlorambucil ratio was 1:1.52 (w:w). This ratio corresponds to a molar ratio of protein to chlorambucil of 1:750 [22]. The pH of the mixture was adjusted to 10.5 with 0.1 N NaOH. The mixture was incubated at 37°C for 10 min and subjected to gel filtration on a column of Sephadex G-25 (coarse) equilibrated in 0.15 M NaCl and 0.01 M Tris–HCl (pH 8.5) at 4°C.

The protein containing fractions were dialysed against PBS at 4°C for 24 hr and stored in quantities of 10 ml at −20°C.

Quantities of the drug and the immunoglobulin used for treatment of tumour bearing mice

After the linking procedure the amounts of (active) chlorambucil per ml and protein per ml were determined.

The amount of protein/ml after the linking procedure with chlorambucil was determined according to the method of Lowry *et al.* [28]. After linking chlorambucil with antitumour antibodies a protein concentration of 0.816 mg/ml and after linking chlorambucil with control antibodies a protein concentration of 0.660 mg/ml were found respectively (Table 1).

The active and the hydrolyzed form of chlorambucil in the complex were measured with a method described by Linford [29]. Samples containing free or physically linked chlorambucil were extracted with ethanol. After centrifugation the supernatant fractions were dried by evaporation under reduced pressure. The residues were dissolved in 0.1 M

Table 1. Experimental groups

Treatment*	Doses/injection		Denoted in Figs. 1–3 as:
	Chlorambucil† (mg)	Immunoglobulin (mg)	
Chlorambucil–anti-HPM-IgG complex	0.193	0.816	Immune complex
Chlorambucil +anti-HPM-IgG	0.195‡	0.738‡	Immune mixture
Chlorambucil +2 hr later anti-HPM-IgG	0.195	0.738	Chl.→immune IgG
Anti-HPM-IgG +2 hr later chlorambucil	0.195	0.738	Immune IgG→Chl.
Chlorambucil–control IgG complex	0.197	0.660	Control complex
Chlorambucil +control IgG	0.195	0.738	Control mixture
Chlorambucil +2 hr later control IgG	0.195	0.738	Chl.→control IgG
Control IgG +2 hr later chlorambucil	0.195	0.738	Control IgG→Chl.
Anti-HPM-IgG	—	0.738	Immune IgG
Control IgG	—	0.738	Control IgG
Chlorambucil	0.195	—	Chl.
Medium	—	—	Medium

*Treatment comprised 5 i.p. injections (1 ml) given with intervals of 24 hr; the first injection was given 24 hr after challenge with 10^7 HPM cells.

†Active form of chlorambucil.

‡When the chlorambucil and the IgG were not complexed the mean values of the doses in the immune and control complex were used.

potassium phosphate buffer (pH 3.6) and extracted with (a) benzene for isolation of the active form of the drug and then with (b) ethylacetate in which the hydrolyzed (inactive) form of chlorambucil is dissolved. The extracts were evaporated under reduced pressure and the residues were dissolved in absolute ethanol. The absorbance of the dissolved residues was measured with the spectrophotometer in the region 230–280 nm with continuous scanning.

The amount of chlorambucil was determined from the absorbance at 258 nm after correction for scattering [29, 30]. As a reference a solution of 30 mg chlorambucil/ml ethanol was used with an absorbance of 1.55 corrected for scattering. It was shown that (a) 0.237 mg active chlorambucil per mg protein was linked after complexing of the drug with anti-HPM immunoglobulins and (b) 0.298 mg active chlorambucil per mg protein was linked after complexing of the drug with control immunoglobulins.

The dose of the drug and/or the protein in the "control" solutions were adjusted to the determined values (Table 1).

The results are in agreement with those of Vennegoor *et al.* [22].

Antibody activity of the anti-serum

Antibody activity of the rabbit-anti-HPM serum was tested in (a) membrane-immunofluorescence and (b) cytotoxicity tests.

Experimental design of the in vivo *experiments*

Groups of 10 BALB/c mice were injected i.p. with 10^7 HPM cells and treated as outlined in Table 1.

Statistics

Mice were observed each day. They were considered to be cured if they were macroscopically free of tumour 120 days after challenge.

The data given in Figs. 1, 2 and 3 were submitted to the Wilcoxon rank-sum test.

RESULTS

Antibody activity of the antiserum

(a) "Membrane immunofluorescence" of the antiserum, the isolated immunoglobulin fraction and chlorambucil antibody complexes with HPM, SL2, BALB/c spleen cells, BALB/c skin cells and DBA/2 spleen cells was performed with the indirect method with goat-anti-rabbit-fluorescein isothiocyanate (GAR-FITC; Nordic, Pharmaceuticals and Diagnostics, Tilburg, The Netherlands).

Fluorescence microscopy showed that HPM could be labelled with anti-HPM serum (titer 1/640), reconstituted freeze-dried anti-HPM IgG (titer 1/160) or with the immune complex (titer 1/64). Further it was shown that the anti-HPM serum showed some crossreaction with DBA/2 derived SL2 lymphoma cells

(titer 1/80), DBA/2 spleen cells (titer 1/40), BALb/c spleen cells (titer 1/80) and BALB/c skin cells (titer 1/80).

Incubation of HPM cells, SL2 or BALB/c skin cells with control serum, control reconstituted freeze-dried IgG, the control complex or GAR-FITC only did not result in labeling of the cells.

(b) Cytotoxicity. It was shown that neither the anti-HPM immunoglobulin nor the control immunoglobulin isolated from the absorbed sera were cytotoxic to: (1) HPM tumour cells, (2) DBA/2 derived SL2 lymphoma cells, (3) BALB/c spleen or lymph node cells.

Even when rabbit complement (which has been described as a more potent complement than guinea pig complement; [31]) was used in the cytotoxicity tests, or when the HPM cells were pretreated with trypsine (0.05–0.02%) and/or 0.02% EDTA at 37°C for 10–30 min no lysis of the HPM cells could be obtained.

The anti-HPM serum showed no anticomplement activity.

In vivo *experiments*

The results of the *in vivo* experiments are given in Figs. 1, 2 and 3. The data given in Fig. 1 show that when BALB/c mice injected i.p. with 10^7 HPM cells were treated with 5 i.p. injections with the chlorambucil (anti-HPM) antibody complex 50% of the mice were macroscopically free of tumour 120 days later. Treatment with (a) a mixture of chlorambucil and anti-HPM antibody or (b) successive injection of the drug and the antibody (or vice versa) were less successful in this system.

The data given in Fig. 2 show that when HPM bearing BALB/c mice were treated with

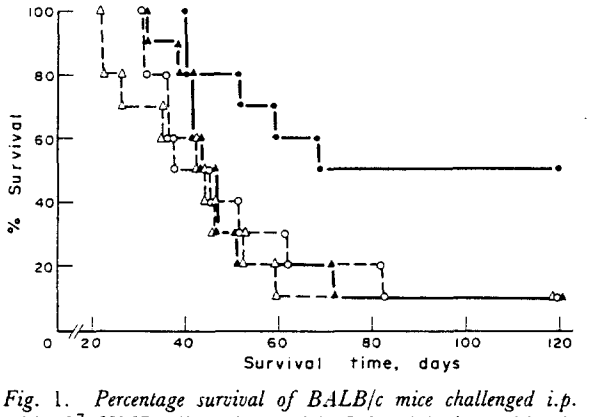

Fig. 1. Percentage survival of BALB/c mice challenged i.p. with 10^7 HMP cells and treated by 5 i.p. injections with: the immune complex (●——●), the immune mixture (○- - -○), immune IgG→Chl. (▲——▲) or with Chl.→ immune IgG (△---△).

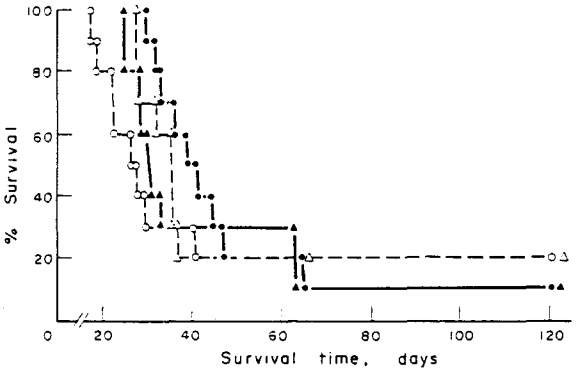

Fig. 2. Percentage survival of BALB/c mice challenged i.p. with 10^7 HPM cells and treated by 5 i.p. injections with: the control complex (●——●), the control mixture (○- - -○), control IgG→Chl. (▲——▲) or with Chl. →control IgG (△---△).

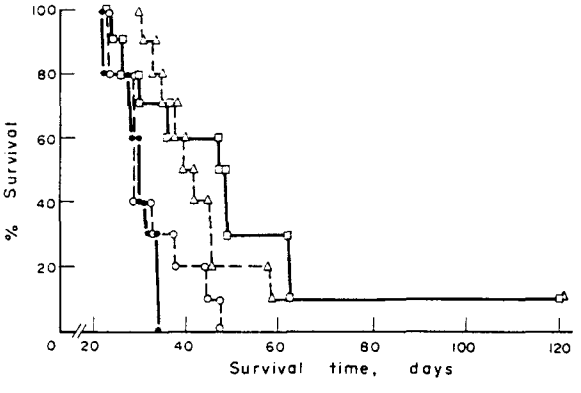

Fig. 3. Percentage survival of BALB/c mice challenged i.p. with 10^7 HPM cells and treated by 5 i.p. injections with: medium (●——●), immune IgG (○——○), control IgG (△---△) or with Chl. (□——□).

the control complex 10% of the mice were macroscopically free of tumour 120 days later. The number of survivors after treatment with (a) a mixture of chlorambucil and control antibody or (b) successive injection of the drug and control antibody (or vice versa) were not significantly different.

Finally it was found that the control antibodies as well as chlorambucil had some antitumour effect of their own since 10% of the mice treated with one of these components were macroscopically free of tumour 120 days later (Fig. 3).

The statistical analysis of the data given in Figs. 1, 2 and 3 showed that the treatment with the immune complex is significantly (5% level) better than treatment with any other solution.

DISCUSSION

Several approaches have been studied to increase the efficacy of currently available

anti-tumour drugs through improved application of these drugs. One approach has been the use of anti-tumour antibodies [6, 8, 13] or other large molecular weight [32, 33] materials as carriers for cytostatic or cytotoxic drugs. Treatment of tumours with cytostatic or cytotoxic agents linked to anti-tumour antibodies offers the possibility of combining the chemotherapeutic potency of the agents with the "homing" activity of the antibodies. In our experiments chlorambucil was physically linked to antibodies according to a method described by Blakeslee and Kennedy [27]. Though it has been shown that cytotoxic drugs can be covalently bound to macromolecules without loss of their biological activity [34, 35] the covalent bond is not a necessity [17, 36] Cumulative energy of multiple ionic and other non-covalent bonds between two molecules has also shown to produce very stable complexes [33, 34]. Another problem encountered in non-covalent linking of chlorambucil to antibody is the possible formation of aggregates of chlorambucil [16]. The presence of these aggregates might lead to overestimation of the amount of active chlorambucil which is physically linked to the antibody.

In our experiments the observed antitumour effect of the immune complex was better than the antitumour effect of (a) a mixture of the two components, (b) antibody followed 2 hr later by chlorambucil, (c) chlorambucil followed 2 hr later by antibody, (d) chlorambucil only or (e) antibody only (Figs. 1–3).

The precise mechanism of increased tumour inhibition by cytotoxic drugs attached to antibodies still remains to be elucidated. As stated by Ghose *et al.* [12, 13] it might be explained on the basis of (a) a synergism between the drug and antibody [8, 19, 21], (b) antibody mediated preferential localization of the drug on tumour cells [37] or (c) both.

With regard to the mechanism of inhibition of tumour growth by the complex our results are of interest since the anti-HPM antibody adhered to the HPM cells, as shown by immunofluorescence, but was not cytotoxic to HPM cells. Experiments were performed to examine the cytotoxicity of the antibody but all results were negative. The positive results with the complex in the *in vivo* experiment suggest that the anti-HPM-antibody in the complex acted simply as a carrier of the chlorambucil.

Although, complexing of chlorambucil with antibody seems to be essential, even in this situation a certain form of synergism cannot be excluded since (a) Segerling *et al.* [38] found that exposure of target cells to cytotoxic drugs renders the target cells more susceptible to antibodies, and (b) Guclu *et al.* [39] stated that "capping" facilitates the transport of cytotoxic drugs across the cell membrane.

The growth rate of a tumour, a characteristic that may reflect both host resistance and inherent growth potential of the tumour cells might also influence the efficacy of the therapy [5]. This is suggested by our recent findings that treatment of SL2 lymphoma bearing-mice (survival time of the recipient mice after i.p. injection of 10^7 SL2 cells is about 10 days) with chlorambucil–anti-SL2 antibody complexes does not result in a prolongation of the survival time of these mice (preliminary results).

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