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(54) Title: TECHNETIUM-ANTIBODY CONJUGATE

(57) Abstract

A conjugate of technetium with a radical having an antigen binding site wherein the technetium thereof is radioactive.

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TECHNETIUM-ANTIBODY CONJUGATE

This invention relates to a technetium-antibody conjugate.

The present invention provides a conjugate of technetium with a radical having an antigen binding site wherein the technetium thereof is radioactive.

The preferred technetium isotope is ^{99m}Tc.

In a particular aspect the present invention provides a technetium-antibody or antibody fragment conjugate which is preferentially absorbed by a tumour cell as compared to a non-tumour cell.

Preferably the conjugation is via a sulphide linkage.

The present invention also provides a compound of formula $Ab\text{-}Y\text{-}S\text{-}NTc(Hal)_3$ where Hal is chlorine, bromine or iodine and including mixed halides, and Y is a conjugating chain and Ab is an antibody radical or a radical having an antigen binding site.

In a preferred instance Y is of the formula

$$-[NH-\overset{\overset{\large X}{\|}}{C}-(\overset{\overset{\large Z}{|}}{C}H)_n]_z$$

wherein Z is H, alkyl, aryl, carboxy,halide hydroxy or amino, n is 1-10, X is NH, O or S and z is O or 1.

Alkyl groups preferably 1 - 6 carbon atoms, aryl groups preferably 5 - 16 carbon atoms.

The present invention also provides compounds of formula

$Ab\text{-}S\text{-}NTc(Hal)_3$

$Ab\text{-}NH\text{-}Y\text{-}S\text{-}NTc(Hal)_3$

wherein Ab, Ab-NH or Ab-S represents an antibody radical or a radical having an antigen binding site and Y and Hal have the meaning given above.

The present invention also provides the intermediate compounds

Ab-SH

Ab-NH-Y-SH

wherein Ab, Ab-NH or Ab-S and Y have the meaning given above.

1 Compounds in accordance with this invention may be
2 produced by taking one of said intermediate compounds and
3 reacting with $TcN(Hal)_4$ wherein Hal has the meaning given
4 above.
5 The intermediate compounds may be formed by
6 a) reducing an antibody to form free sulphydryl groups.
7 Such reduction may be effected in a number of ways but it is
8 presently preferred to use dithiothreitol (DTT),
9 b) reacting an antibody succinimidyl pyridyldithio-
10 propionate (SPDP) or an analogue thereto appropriate to the
11 compound desired to obtain an antibody conjugate containing
12 a -S-S-group, reducing the conjugate to form a -SH group,
13 c) using S-acetylmercaptosuccinic anhydride (SAMSA) or
14 SH introducing compounds to produce a side chain on an
15 antibody containing a -S-linkage and reducing to form a -SH
16 group,
17 It is preferred that said radical is an antibody.
18 The antibody may be a monoclonal antibody. Antibodies
19 useful in the present invention included those showing
20 specificity for breast, brain, melanoma, lung, pancreas and
21 colon tumours.
22 The antibody may be an intact immunogobulin or a
23 fragment of an immunogobulin maintaining a sufficiency of an
24 antigen binding site such that it is preferentially absorbed
25 by a tumour cell as compared to a non tumour cell.
26 Thus, in addition to whole antibodies, it is also
27 possible to utilize $F(ab')_2$ and F(ab') fragments.
28 Still further antibody polymers such as antibody
29 pentamers IgM and derivatives of these such as immunogobulin
30 monomers may be used.
31 Also useable are IgG_{2a}, IgG_{2b}, IgG_1 and IgG_3.
32 The compounds of this invention may be combined with
33 pharmaceutically acceptable carriers.
34 The mode of administration of the compounds of this
35 invention will be as selected. In particular, the compounds
36 of this invention may be administered intravenously,
37 intraperitonealy, intrapluraly, intrapericardialy,
38 intracerebospinal fluid and subcutaneously.

1 The technetium-antibody conjugates of the present
2 invention may be formed into pharmalogical compositions with
3 appropriate pharmaceutically acceptable diluents.
4 The technetium-antibody conjugates of the present
5 invention are useful for in vivo detection of tumours such
6 as by immunoscintigraphy.
7 Part A
8 Radioactive isotopes of technetium coupled to MoAb have
9 been used by us in the search for specific methods of
10 diagnosing small tumours. In this light we have
11 successfully located tumours in both mouse and man, and
12 antibodies have been administered either intravenously or
13 subcutaneously, or by other routes. It is clear that
14 radiolabelled MoAb can indeed localise in tumours *in vivo*
15 and with the use of computer assisted tomography, with
16 subtraction for non-specific effects, this method can then
17 be utilized for the *specific* detection of tumours - both
18 primary and secondary. However, there are problems of
19 specific activity, specificity and high blood background
20 which need attention before this technique can be accepted
21 as a useful diagnostic tool. Major advances in the
22 diagnostic radiolocalization of tumours should result from
23 the production of better MoAb, better methods of
24 radiolabelling and finally, design of methods to reduce the
25 background provided by circulating radiolabelled antibodies.
26 It is expected that more specific antibodies will become
27 available with time. We have shown that the use of second
28 antibody (anti-immunoglobulin), is able to clear the
29 circulating pool of antibody, and thereby significantly
30 lower the background.
31 Prior use by us of ^{131}I, or a combination of ^{131}I, or a
32 combination of ^{131}I and ^{125}I in experimental models has
33 shown that ^{125}I cannot be used in man because of high tissue
34 attenuation but there are however serious drawbacks with the
35 use of ^{131}I. This nuclide provides a poor quality image, it
36 produces significant radiation exposure due to its beta
37 emissions and has a short biological half life - presumably
38 due to the de-iodination of the MoAb. The use of ^{99m}Tc for

labelling MoAb as in this invention offers several advantages: it has a reasonably short half life; it is cheap, easy to produce, and is readily available. The isotope has an optimal gamma energy (140keV) for detection with currently available scintigraphic instrumentation and produces very little radiation exposure to patients undergoing scanning procedures.

Materials and Methods

Mice: Mice used were: RF/J, C57BL/6 and (C57BL/6 x BALB/c)F_1 (=BCF_1) bred in our colony. Athymic, BALB/c mice (*nu/nu*) were obtained from the Royal Dental Hospital (Melbourne, Australia).

Tumour Cell Lines: Two tumour cell lines were used: one, the E3 clonal variant of the thymoma ITT(1)75NS(1) which was obtained by three successive rounds of fluorescent activated cell sorting of ITT(1)75NS cells stained with monoclonal Ly-2 antibodies and selected for the most fluorescent 1% of cells. The murine cell line E3 was maintained *in vitro* in DME supplemented with 10% heat inactivated newborn calf serum (Flow Laboratories, Sydney, Australia), 2mM glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 IU penicillin/ml and 100 mg streptomycin/ml (Glaxo Laboratories, Melbourne, Australia). E3 cells were washed twice in DME (without additives) and twice in DME containing 0.5% BSA and used in the *in vitro* binding assays. The E3 cell line was maintained *in vivo* by the passaging of cells from ascites fluid produced in BCF_1 mice. Ascites were washed in DME and PBS, solid tumour grew after the s.c. injection of 10^6-10^7 cells. The second cell line used was a human colonic carcinoma, COLO 205 (2), maintained in culture with RPMI containing the same additives; adherent cells were harvested with 0.125% trypsin (Commonwealth Serum Laboratories, Australia) washed with RPMI and injected s.c. into nude mice, where tumours appeared after the injection of $2 \times 10^6 - 1 \times 10^7$ cells.

MoAb: Two MoAb were used: (i) anti-Ly-2.1 (IgG2a), an antibody raised against the murine alloantigen Ly-2.1 (3); and (ii) 250-30.6 (IgG2b), an antibody to human colonic

1 secretory epithelium (4). The MoAb were isolated from
2 ascitic fluid by precipitation with 40% ammonium sulphate,
3 followed by dissolution in 0.01 M Tris buffer pH 8.0 and
4 extensive dialysis against the same buffer and further
5 purified by affinity chromatography using protein-A
6 Sepharose (Pharmacia Inc., Piscataway, NJ, U.S.A.)and purity
7 was confirmed by gel electrophoresis and antibody activity
8 assayed by a rosetting test (5).

9 <u>^{99m}Tc Labelling of MoAb):</u> Sodium Pertechnetate
10 Injection B.P. produced from fission product chromatography
11 generators was used for all preparations. Generators were
12 obtained either from the Australian Atomic Energy Commission
13 (Lucas Heights, Sydney, Australia), or from Mallinckrodt
14 Inc. (St.Louis, MO.USA). MoAb were labelled with ^{99m}Tc
15 using two methods - the new method described herein, and a
16 method using stannous chloride.

17 (a) Labelling using $^{99m}TcNCl_4^-$:$^{99m}TcNCl_4^-$ was prepared as a
18 dry salt residue as described in detail in (9). For
19 labelling, the MoAb was first reduced with the addition of
20 DTT (20microl, 115mg/ml in PBS) to 200microg of MoAb (1
21 mg/ml in PBS) and allowing the mixture to stand at room
22 temperature for 30minutes when the reduced MoAb was
23 separated from DTT by gel chromatography using 0.1 M sodium
24 acetate pH 4.0 as eluant on an 8cm x 1cm column of Biogel P-
25 6 (Biorad Laboratories, Richmond, U.S.A.). The fractions (1
26 ml) containing the protein peak were added to the dried
27 $^{99m}TCcl_4^-$ salt residue and the mixture brought to pH 3.0
28 with 0.2M hydrochloric acid; after 2 minutes at room
29 temperature, 0.1M sodium phosphate was added and the pH
30 adjusted to 7 by the careful addition of sodium hydroxide.
31 Purification of the labelled MoAb was then achieved by gel
32 chromatography with a Sephadex G-25 column (PD-
33 10,Pharmacia).

34 (b) Stannous Chloride Reduction Method: Stannous chloride
35 (20-200 microg) was added to 200 microg MoAb (1mg/ml), 4-6
36 mCi of pertechnetate was added, and the mixture allowed to
37 stand at room temperature for 30 minutes. The labelled MoAb
38 was purified by gel filtration on a PD-10 sephadex column.

1 Radioiodination of MoAb: MoAb (100 microg, 1mg/ml)
2 were labelled using the chloramine-T method (6): 2.5mCi of
3 carrier-free $Na^{125}I$ (Amersham International Ltd., Amersham,
4 England) and 3 microl of chloramine-T (1 mg/ml) were mixed
5 with the protein for 2 minutes at room temperature and the
6 reaction then terminated by the addition of 3 microl of
7 sodium metabisulfite (2.4 mg/ml). Iodinated MoAb was
8 separated from free iodine by gel filtration using a PD-10
9 column.
10 Serological Analysis: A binding assay was developed to
11 determine the stability and specificity of the ^{99m}TcN-MoAb
12 complexes. MoAb complexes were tested in one of two ways -
13 either a) using one MoAb and two cell lines; or b) using two
14 different MoAb and one cell line - both MoAb being labelled
15 identically, one being reactive with the cell line, the
16 other non-reactive. Polyvinyl chloride 96 well plastic
17 plates (Pynatech Laboratories, Inc., Alexandria, Va) were
18 washed with 1% bovine serum albumin (BSA) in PBS. In this
19 assay either the number of cells or the quantity of MoAB
20 could be kept constant while the other was varied. Either:
21 (1) after serial dilution of 25 microl of the labelled MoAb,
22 $1x10^7$-$2x10^7$ target cells (either tumour cell lines or
23 thymocytes) suspended in PBS were added to each well; or (2)
24 different cell numbers (10^8- 2 x 10^4) contained in the same
25 volume of the target cells were added and an equal amount of
26 radiolabelled MoAb was then added to each well. In both
27 assays the contents were mixed and the plate incubated on
28 ice for 30 minutes; after 3 washes, the plates were dried
29 and the cell pellets were counted for one minute in a gamma
30 counter. All assays were performed in duplicate.
31 Biodistribution: Nude mice bearing COLO 205 xenografts
32 or BCF_1 mice bearing the E3 thymoma were used. The first
33 study compared the distribution of two ^{99m}TcN-MoAb in BCF_1
34 mice; groups of 4 mice were sacrificed at 20 hrs, 30 hrs and
35 35 hrs after the injection of labelled MoAb. The second
36 study compared the binding of a ^{99m}TcN-MoAb complex to two
37 different tumours - the E3 thymoma and COLO 205 xenografts.
38 In these studies, the biodistribution of ^{99m}Tc label was

1 determined by gamma counting of blood, heart, lungs, spleen,
2 stomach, intestine, kidneys and tumour from these mice. The
3 distribution of isotope is reported as a localization ratio
4 (tissue (cpm/g)/blood (cpm/g)). All mice received
5 approximately 115 microCi of ^{99m}Tc activity (approximately
6 10 microg of protein) by tail vein injection.

7 Imaging: BCF_1 mice bearing the E3 thymoma (0.3 -
8 1.0cm) were given i.v injections of either approximately 115
9 micro Ci of ^{99m}TcN-labelled anti-Ly-2.1 (specific MoAb) and
10 200 micro Ci (4microCi/micro g) of ^{125}I labelled anti colon
11 (non-specific MoAb) or 115 micro Ci of ^{99m}Tc-labelled anti
12 colon MoAb. Twenty-seven hours after injection mice were
13 anaesthetised by intraperitoneal injection of 4% chloral
14 hydrate (0.01 ml per g body weight). Vertical views of the
15 mice were taken using a Toshiba GC 402A gamma camera and a
16 low energy parallel hole collimator. A setting of 50 keV
17 with an 80% window and 140 keV with a 20% window was used to
18 image the ^{125}I and ^{99m}Tc photons respectively. Data were
19 stored in digital form by an MDS Modumed computer.

20 RESULTS

21 The study was conducted in three phases: the
22 establishment of the conditions for the coupling of
23 $^{99m}TcNCl_4^-$ to MoAb; the testing of the stability of the
24 ^{99m}TcN-MoAb complexes *in vitro*; and the measurement of the
25 distribution of the complexes *in vivo* to determine whether
26 tumours could be detected specifically.

27 ^{99m}Tc-MoAb ($SnCl_2$): ^{99m}Tc labelled anti-Ly-2.1 MoAb
28 was tested in a binding assay using thymocytes from two
29 strains of mice - RF/J (Ly-2.1$^+$) and C57BL/6 (Ly-2.1$^-$). In
30 this assay the amount of labelled MoAb added to each well
31 was kept constant, the cell number varied (10^5-10^8 cells per
32 well). There was clearly no noticeable difference in the
33 uptake of radioactivity by the two cell types i.e. the
34 ^{99m}Tc-MoAb complexes bound to non reactive and reactive
35 cells equally. Varying the amount of $SnCl_2$ used in the
36 reduction procedure had no effect on the result. This is
37 shown in Fig.1 which shows the binding of ^{99m}Tc labelled
38 anti-Ly-2.1 prepared by $SnCl_2$ reduction of RF/J and C57BL/6

thymocytes. The amount of radioactivity incorporated as a function of cell number is shown.

<u>^{99m}TcN-MoAb:</u> An alternative method of labelling was designed, utilising the unique property of $^{99m}TcNCl_4^-$ to form a stable covalent linkage to sulfur atoms. MoAb were partially reduced with DTT to generate free sulfhydryl sites and mixed with the $^{99m}Tc\ Cl_4^-$, leading to the formation of ^{99m}TcN-MoAb complexes. The ^{99m}TcN-MoAb complexes were then tested in different serological assays to determine whether the labelling procedure damaged or altered the binding or specificity of the MoAb, and whether the complexes formed were stable.

The binding curve obtained when ^{99m}TcN-labelled Ly-2.1 MoAb was incubated with thymocytes from mouse strains RF/J (Ly-2.1$^+$) and C57BL/6 (Ly-2.1$^-$) is shown in Fig.2 which shows the specific binding of anti-Ly-2.1 labelled with $^{99m}TcNCl_4^-$ on RF/J and C57BL/6 thymocytes; Amount of radioactivity bound as a function of antibody in the reaction mixture. There was clearly a major difference in the binding of the labelled MoAb to the reactive cells (RF/J) when compared to the non-reactive cells (C57BL/6) with specific ratio (=cpm RF/J / cpm C57BL/6) of greater than 20. Thus with the use of the one antibody and two difference sources of cells the procedure used to couple $^{99m}TcNCl_4^-$to MoAb produced a stable complex which bound only reactive target cells as shown in Fig.2.

The results are clearly superior to those obtained with the complex formed with the use of $SnCl_2$. In this respect, reference is made to Fig.1 which shows the binding of ^{99m}Tc labelled anti-Ly-2.1 with $SnCl_2$ reduction on RF/J and C57BL/6 thymocytes; amount of radioactivity incorporated as a function of cell number. In the second assay, two different MoAb, one directed against colonic secretory epithelium (250-30.6) and the second, the anti-Ly-2.1 MoAb, were labelled with $^{99m}TcNCl_4^-$ under identical coupling conditions and the two complexes were tested for their ability to bind to the murine T cell thymoma E3;, which is Ly-2.1$^+$ but does not react with the anti-colon MoAb (250-

1 30.6). Again the specific MoAb complex ^{99m}Tc Ly-2.1 bound
2 more efficiently than the ^{99m}TcN-anti-colon complex as shown
3 in Fig.3 with a specific ratio of 10. Fig.3 shows the
4 specific binding of $^{99m}TcNCl_4^-$labelled anti-Ly-2.1 and
5 anti-colon Mo-Ab on ITT(1) 75NS E3 target cells. Thus in
6 these assays, it appeared that a stable bonding of ^{99m}Tc to
7 MoAb had been produced, so that only the binding of antibody
8 to the appropriate target cell was detected.

9 Stability of ^{99m}TcN-MoAb: Aliquots of ^{99m}Tc labelled
10 Ly-2.1 MoAb were stored at 4°C for 20 hrs and then tested in
11 binding assays with RF/J and C57BL/6 thymocytes. No loss of
12 binding reactivity was observed when the binding curve (as
13 shown in Fig 4) was compared with that obtained at 6 hrs (as
14 in Fig.2). Fig.4 shows the binding of anti-Ly-2.1 labelled
15 24 hours previously with $^{99m}TcNCl_4$, on RF/J and C57BL/6
16 thymocytes.

17 Effect of ^{99m}Tc Concentration on the Activity of
18 ^{99m}TcN-MoAb complexes: At the time when ^{99m}Tc is obtained
19 from a ^{99m}Tc generator, there is approximately 0.7 micro g
20 ^{99m}Tc/ml of ^{99}Tc eluted (8). As the number of labelled
21 binding sites on the antibody molecule is determined by the
22 chemical quantity of technetium present, the effect of
23 labelling with increased quantities of ^{99m}Tc may be studied
24 by the addition of $^{99m}TcCl_4^-$ carrier to the $^{99m}TcO_4$-used for
25 labelling. The addition of 2 micro g ^{99}Tc to the reaction
26 mixture was thus equivalent to increasing the ^{99m}Tc activity
27 used by a factor of 200. This approach was adopted to avoid
28 the radiation hazards associated with the handling of high
29 levels of activity and to overcome "dead-time" problems
30 which would arise in the gamma counting of very high
31 activities.

32 The binding curves obtained using ^{99m}TcN-anti-Ly-2.1
33 containing added ^{99}Tc carrier are shown in Fig.5. Fig.5
34 shows the binding of two ati-Ly-2.1 conjugates - one
35 containing added Tc carrier, the other carrier free on
36 RF/J and C57BL/6 thymocytes. The binding observed to RF/J
37 (Ly-2.1$^+$) and C57Bl/6 (Ly-2.1$^-$) cells was the same as that
38 observed for the preparation containing no added ^{99}Tc as in

1 Fig.2 and again, a specificity ratio of greater than 20 was
2 observed. Hence these results show that increasing the
3 specific activity of the preparation by a factor of 200
4 would not effect the binding specificity of the MoAb.
5 Biodistribution: The *in vivo* localization and
6 biodistribution of ^{99m}Tc-MoAb complexes was examined by
7 injecting mice with ^{99m}Tc-MoAb and determining the relative
8 amounts of radiolabel accumulated in the tumour or the
9 tissue. These results were used to calculate the
10 localization ratio derived as follows: tissue (cpm/g) /
11 blood (cpm/g).
12 In the first study, two groups of 16 BCF_1 mice bearing
13 the E3 (Ly-2.1$^+$) tumour (0.23 - 1.11g) were injected i.v.
14 with either ^{99m}Tc-Ly-2.1 or ^{99m}Tc-250-30.6 MoAb - each mouse
15 received 115 micro Ci ^{99m}Tc and 10 micro g MoAb. Four mice
16 from each group were sacrificed at different time intervals
17 after injection (20, 30.5, 35 hrs) and the distribution of
18 the two MoAb determined. After 20 hours, the tumour
19 localization was observed to be 3 times greater for the
20 specific MoAb than that observed for the non specific
21 antibody Ly-2.1) with the localization ratios in liver,
22 spleen and kidney being less than or similar to that of
23 blood. The non-specific antibody (250-30.6) the
24 localization ratio of the liver, spleen and kidney were
25 observed to be higher than that of the blood and at 30.5
26 hours the liver localization ratio was 5 times greater than
27 that of blood - the reason for this high ratio is unknown,
28 but may be due to the different reactivity of the MoAb.
29 In the second study, the localization of ^{99m}Tc-Ly-2.1
30 was compared in two different tumours -nude mice bearing
31 Colo 205 xenografts (Ly-2.1$^-$) or the E3 thymoma (Ly-2.1$^+$)
32 were used. After 20 hours the E3 thymoma (Ly-2.1$^+$) was
33 observed to take up to 4 times more radioactivity than the
34 Colo 205 xenografts (Table 1) and there was at least 4 times
35 more radioactivity in the tumour than in other tissues -
36 except the liver. The ^{99m}Tc-MoAb complexes could
37 specifically localise to tumours *in vivo*.
38 Radio-Imaging

A. Nuclear image obtained after the injection of ^{99m}Tc-anti-Ly-2.1 into a mouse bearing the E3 thymoma on the right thigh (as visualized).

B. Nuclear image obtained after the injection of ^{99m}TcN-anti-Ly-2.1 into a mouse hosting a thymoma on its left anterior side and ^{125}I-anti-colon.

C. ^{125}I nuclear image of the same mouse as in B.

In the initial imaging experiments, tumours (0.23-1.2g) could be visualized with the use of a small animal scanner as early as 2 hrs after injection of the specific ^{99m}TcN-MoAb (results not shown) the visualization became well defined with time. The mouse in B. had an E3 tumour (1.0cm in diameter) which was easily seen as a distinct single entity on the right hind leg. The tumour was dissected and found to have a localization ratio (tumour to blood approximately 2.0). Radioactivity in this image is also concentrated in the central region of the mouse, indicative of significant distribution of antibody to large vascularized organs such as liver, lung and heart; a phenomenon that tends to obscure visualization of small tumours. The second image B. was of a mouse with a much smaller thymoma (0.4cm in diameter) on the left anterior side and again the tumour is again clearly visualized. The tumour was dissected and found to have a localization ratio of approximately 1-2. At the time of injection with ^{99m}Tc-MoAb this mouse was also injected, C., with a non-reactive MoAb (250-30.6) labelled with ^{125}I, however scanning failed to localize the tumour with this MoAb. It was noted that the tumours were readily visualized and that the contribution of the reactive MoAb to the overall blood pool radioactivity did not obscure the visualization of the tumour and consequently a computer assisted subtraction of the image provided by the non-reactive MoAb (^{125}I labelled) - the control blood pool was not required.

Discussion

Immunoscintigraphy, with the use of radiolabelled MoAb is a new method for the *in vivo* detection of tumours and thus cancers of colon, breast and other tissues have been

1 detected with some degree of success. However there is not
2 a marked increase in the detection rate of tumours, except
3 in a few cases only previously identified tumours could be
4 detected and clearly the sensitivity of the procedure needs
5 to be increased. As the specificity and sensitivity is
6 determined by the ratio of the amount of radiolabelled MoAb
7 bound versus the background blood pool, the ways of
8 increasing this ratio are either to directly increase the
9 primary signal (by altering MoAb and/or isotope) or to
10 reduce the background. We are adopting both approaches
11 through the use of multiple MoAb and MoAb fragments (Fab or
12 $F(ab')_2$). In this manuscript we report on the advantages of
13 using ^{99m}TcN-MoAb to detect tumours. In this study a new
14 method of coupling $^{99m}TcNCl_4$ to MoAb and the subsequent use
15 of these complexes to detect tumours in vivo is described.
16 As previously indicated, ^{99m}Tc offers a number of advantages
17 for radioisotopic localization studies in patients, as it
18 has a short half life (6 hrs) and thus limits the radiation
19 exposure to patients and has an optimal gamma energy (140
20 keV) ideal for currently used scintigraphic instrumentation.
21 $^{99m}TcO_4^-$ from a portable generator and must be reduced prior
22 to coupling with MoAb. Many methods have been described for
23 the reduction of pertechnetate; these procedures generally
24 lead to the reduction of pertechnetate to the Tc (IV) or TC
25 (V) oxidation state. At present, the most frequently used
26 reducing agent for preparation of ^{99m}Tc labelled compounds
27 is $SnCl_2$. Problems have been experienced with this agent
28 when used for labelling MoAb, such as hydrolysis,
29 instability towards oxidation and competition of Sn for
30 binding sites. Indeed, in our hands pertechnetate reduced
31 with $SnCl_2$ readily bound to MoAb but such complexes showed
32 no specificity when tested in our _in vitro_ binding assay or
33 when tested _in vivo_. Because of the problems experienced
34 with the ^{99m}Tc labelling of MoAb with the use of $SnCl_2^-$ we
35 have used ^{99m}Tc NCl_4 - to produce ^{99m}TcN-MoAb by a
36 substitution reaction. Important features of our study have
37 been to show that MoAb may be labelled without loss of
38 activity to yield a highly specific complex which retains

1 its stability for at least 24 hours and which yield superior
2 results when tested in vivo by immunoscintigraphy. Tumours
3 could be visualised as early as two hours after injection,
4 and small tumours (approximately 0.4cm in diameter) located
5 near large vascular organs could be visualised without the
6 need for blood pool subtraction.

7 The complexes formed with the use of $^{99m}TcNCl_4^-$ are
8 clearly different to those made using $SnCl_2$ as the presence
9 of the nitrido group attached to Tc modifies the chemical
10 behaviour of the Tc atom and makes it more favourable for
11 co-ordination with certain ligands. Ligands which bind
12 through sulfur atoms form more stable complexes with the TcN
13 core than do ligands binding through nitrogen. In our
14 initial experiments, attempts were made to bind the TcN
15 group directly to amino groups of the MoAb and while ^{99m}TcN-
16 labelling of MoAb was achieved, there was considerable loss
17 of specificity. To utilise the known preference of the TcN
18 group for sulfur atoms, we developed a partial reduction
19 procedure used for the coversion of disulfide linkages to
20 sulfhydryl residues. Such an approach was also attractive
21 because it was known that the sulfhydryl groups involved in
22 the coupling were likely to be distant from the sites
23 responsible for antibody binding. Thus $^{99m}TCNCl_4^-$ was
24 prepared in a stable dry form without the presence of any
25 contaminating metal ions and was successfully complexed to
26 partially reduced MoAb in a simple one step procedure that
27 resulted in a stable covalent complex which retained MoAb
28 activity. It should be noted that the reduction step
29 critical to this procedure used DTT to produce sulfhydryl
30 residues on the MoAb. The chemical stability and activity
31 of $^{99m}TcNCl_4^-$ complexes was determined in several
32 serological assays which involved MoAb reactive and non-
33 reactive cells; either one MoAb, two different target cells
34 or conversely, two MoAb and one call target. In all studies
35 specific binding of radiolabelled MoAb to target cells was
36 demonstrated, the complexes were not non specifically
37 "sticky" nor unstable with the release of ^{99m}Tc to bind to
38 other non reactive target cells.

1 *In vitro* studies have shown that $^{99m}TcNCl_4^-$ may be used
2 to produce chemically stable MoAb complexes that retain
3 their activity for at least 24 hours. Furthermore these
4 complexes may be prepared at a clinically useful specific
5 activity without any changes in the *in vitro* properties.
6 For example, it was possible to increase the amount of ^{99m}Tc
7 bound to b 200 fold without affecting MoAb activity (text
8 fig.5).
9 An important finding obtained in the study was to
10 demonstrate that ^{99m}TcN-MoAb complexes localized to tumours
11 *in vivo*.
12 With the use of nuclear imaging equipment large tumours
13 (0.8 - 1.1cm in diameter) could be easily visualised (A.)
14 but the ultimate sensitivity of this technique lay in the
15 detection of small tumours (0.3 - 0.6cm in diameter) that
16 were located near vascular organs, such tumours being
17 detected without the requirement of a blood pool subtraction
18 (B.). Incidentally, the same mouse received a simultaneous
19 injection of a ^{125}I labelled non-reactive MoAb and
20 subsequent scans could not visualize the same tumour (C.).
21 Similar results were obtained for mice scanned with a non-
22 reactive ^{99m}TcN-MoAb, where tumours (0.6 - 1.5cm in
23 diameter) could not be visualized.
24 Our studies showed that the $^{99m}TcNCl_4^-$ complex could be
25 successfully coupled to MoAb, in a simple one step procedure
26 that resulted in stable covalent complexes which retained
27 MoAb activity. An alternative ^{99m}Tc labelling procedure
28 described in the literature involves the coupling of DTPA to
29 MoAb prior to the coordination of reduced technetium. There
30 are however several difficulties with this procedure. As
31 metal ions are able to compete with ^{99m}Tc for the DTPA
32 coordination sites, reduction systems using heavy metals are
33 thus undesirable. For this reason sodium dithionite has
34 found favour as a reducing agent for this system.
35 Hydrolysis of reduced Tc still remains a problem. Another
36 difficulty is the limitations produced by the number of DTPA
37 molecules that can be coupled to the MoAb before activity is
38 affected. This leads to a restriction on the specific

activity of the labelled MoAb. Our method eliminates the need for DTPA coupled MoAb and the $^{99m}TcNCl_4^-$ used for labelling is free of any heavy metal contamination, thus enabling high specific activity to be obtained.

Thus $^{99m}TcNCl_4^-$ monoclonal antibody can be simply produced and have high activity for specifically localizing tumours both *in vitro* and *in vivo*. At present we consider the coupling method to be superior to other methods of coupling ^{99m}Tc to antibody and the immunoscintigraphic findings to be superior to that obtained with radiolabelled iodine. On this basis we are now conducting a chemical trial to determine the clinical usefulness of the new reagent.

TABLE 1

Biodistribution and Localization of a ^{99m}Tc radiolabeled anti-Ly-2.1 in BCF_1 mice bearing the E3 thymoma and nude mice bearing COLO 205 tumor xenografts.

Tissue	Localization Ratio		E3/COLO 205 Ratio
	ITT(1)E3	COLO 205	
Blood	1.0	1.0	1.0
Tumor	1.23	0.30	4.10
	(.39 - 1.11g)	(0.5 - 1.5g)	
Stomach	0.08	0.2	0.04
Spleen	0.59	0.60	0.98
Kidney	0.77	0.76	1.01
Heart	0.33	0.39	0.85
Liver	0.84	0.56	1.50
Lung	0.38	0.42	0.90
Intestine	0.09	0.15	0.60

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Part B

We now describe the use of the above method to label a panel of MoAb and these have been tested to show that the procedure established has general use, and can be used to label all subclasses of MoAb with ^{99m}Tc with retention of immunoreactivity; the complexes formed can be used to localize tumors in vivo.

MATERIALS AND METHODS

Mice: RF/J, CBA, AKR, C57BL/10(B10), BALB/c, C57BL/6(B6), and (C57BL/6xBALB/c)F1 (B6CF1) mice were bred

1 in our colony.
2 Tumor Cell Lines: Human tumor cell lines (CEM and
3 Bordin - an EBV induced B cell line) were cultured in RPMI
4 1640 medium with L-glutamine. BW5147 and several clonal
5 variants (E3,D1) of the murine thymoma ITT(1)75NS (20) were
6 cultured in DME with L-glutamine. The clonal variant
7 ITT(1)75NS.E3 (E3) was maintained by serial passage in
8 ascitic fluid in (B6CF1) mice. For imaging experiments 10^6 -
9 10^7 cells injected subcutaneously into B6CF1 mice and
10 reached a size of 0.5-1.0 cm in diameter prior to
11 experimentation.
12 Monoclonal Antibodies:
13 The details of MoAb are shown (Table 1). IgM antibodies
14 were isolated from ascitic fluid by dialysis against water
15 at 4°C, after which the precipitate was collected and
16 resuspended in phosphate buffered saline (PBS, pH 7.3): IgG
17 antibodies were prepared by precipitation with 40% ammonium
18 sulfate $[NH_4(SO_4)_2]$, followed by dissolution of the
19 preparation in 0.01 M Tris buffer (pH 8.0); after dialysis
20 against the same buffer, the IgG fraction was further
21 purified by either: (i) adsorption onto Protein-A-Sepharose,
22 washing with PBS and eluting with either 0.2 M glycine-HCl
23 (pH 2.8) or citrate buffers (pH 5.0, 4.0, 3.0) and
24 neutralization with saturated Tris, after which antibodies
25 were dialyzed against PBS; or (ii) ion-exchange
26 chromatography on DEAE-Sephacel and with elution using a
27 linear gradient of 0.5 M NaCl in 0.01 M Tris buffer (pH
28 8.0). The purity of the antibody (>90%) was confirmed either
29 by high pressure liquid chromatography (HPLC) or gel
30 electrophoresis, and antibody activity assayed by a
31 rosetting test (29) or by the immunoperoxidase method on
32 tissue sections (27).
33 Preparation of $^{99m}TcNCl_4^-$ - and Labelling of MoAb:
34 $^{99m}TcNCl_4^-$ was prepared as a dry salt residue (18). Sodium
35 azide (15-20 mg) was added to 1 ml sodium $^{99m}TcO_4^-$, produced
36 from a fission product chromatography generator, in 6-7 mls
37 of concentrated hydrochloric acid (specific gravity, 1.18).
38 The solution was refluxed for 5 minutes to destroy excess

1 azide and then evaporated to dryness in a rotary evaporator.
2 MoAb were labeled with $^{99m}TcNCl_4^-$ by one of two procedures:
3 (i) 100-200mug of MoAb (1 mg/ml) was directly added to the
4 dried $^{99m}TcNCl_4^-$ salt residue, or (ii) a modified version of
5 the method previously described (19) where 20 mul of
6 dithiothreitol (DTT, 115 mg/ml) was added to 200 mug MoAb (1
7 mg/ml) and the solution allowed to stand for 30 minutes at
8 room temperature; it was then transferred to Biogel P6 to
9 remove unreacted DTT and the column eluted with 0.1 M sodium
10 acetate (pH 4.0). The protein fraction (1.5 mls) was added
11 to the dry $^{99m}TcNCl_4^-$ residue, reacted for 2 minutes at room
12 temperature prior to adjusting the pH to 7 with sodium
13 hydroxide. Prior to use, the ^{99m}TcN- labeled MoAb was
14 purified by passage over Sephadex G-25 (PD-10) and
15 sterilised using a 0.22 mum membrane filter.
16 Serological Analysis:
17 In vitro cell binding studies were performed on
18 cultured tumor cell lines or mouse thymocytes (19). ^{99m}TcN-
19 MoAb complexes were tested in one of two ways: (i) using one
20 MoAb and two different target cells; or (ii) using two
21 different MoAbs and one target cell line. The ability of the
22 MoAb to bind to target cells was assessed after each step.
23 In this assay cultured tumor cells or thymocytes ($3x10^5$)
24 were incubated for 30 minutes on ice with one of the
25 following: (i) untreated MoAb; (ii) DTT treated MoAb; or
26 (iii) ^{99m}TcN- labeled MoAb. The cells were then washed 3
27 times with PBS (0.5% BSA), resuspended in PBS and then
28 treated with iodinated sheep anti-mouse immunoglobulin
29 (^{125}I-SAM) for 30 minutes on ice. The cells were then washed
30 3 times with PBS (0.5% BSA) to remove unbound ^{125}I-SAM and
31 the amount of ^{125}I-SAM bound determined.
32 Immunoscintigraphy:
33 Mice bearing E3 tumors were used in two studies. The
34 first compared two identically labeled different MoAb and
35 one tumor; the second compared a specific MoAb in mice
36 bearing several tumors. B6CF1 mice bearing the E3 thymoma
37 (0.5 - 1.0cm in diameter) were given intravenous injections
38 of approximately 115muCi (12muCi/mug) of ^{99m}TcN- labeled

1 anti-Ly-2.1 (specific MoAb) or anti-Ly-1.1 (nonspecific
2 MoAb). Each animal was given an intraperitoneal injection of
3 4% chloral hydrate (0.01 ml/g body weight) imaged 4-28 hours
4 after injection. Vertical views of the mice were taken using
5 a Toshiba GC 42A gamma camera and a low energy parallel hole
6 collimator using a setting of 140 keV with a 20% window to
7 image the ^{99m}Tc photons. Data were stored in digital form by
8 a MDS modumed computer.

9 RESULTS

10 Radiolabeling of MoAb with $^{99m}TcNCl_4^-$: After
11 radiolabeling, unbound reaction products were removed by
12 passage of the final reaction mixture through a gel
13 permeation column of Sephadex G-25 (PD-10). The yield of
14 ^{99m}TcN passing through the column was then a measure of the
15 success of radiolabeling and typical yields were 80-90%. (A
16 typical example of the elution profile is shown in Figure
17 6). It was noted that 98-99% of the radioactivity present in
18 the protein fraction could be precipitated with
19 trichloracetic acid (TCA).

20 Analysis of $^{99m}TcNCl_4^-$ complexed to amino groups: The
21 methods of complexing $^{99m}TcNCl_4^-$ to MoAb were evaluated in
22 serological assays to determine whether the labeling
23 procedure used damaged or altered the binding or specificity
24 of the MoAb. $^{99m}TcNCl_4^-$ complexed directly to amino groups
25 of Ly-2.1 MoAb was tested in a binding assay using
26 thymocytes from 2 strains of mice: RF/J (Ly-2.1+) and B10
27 (Ly-2.1-). ^{99m}TcN-anti-Ly-2.1 achieved almost identical
28 binding to both cell types (Figure 7a), with a specific
29 ratio (cpm bound RF/J / cpm bound B10) of approximately 1.2.
30 In a second assay $^{99m}TcNCl_4^-$ directly bound to 2 different
31 MoAb: anti-Ly-2.1 reactive with the E3 cell line, the other
32 nonreactive (anti-colon carcinoma) produced a specific ratio
33 (cpm anti-Ly-2.1 bound / cpm anti-colon bound) of 2-3
34 (Figure 7b). The conclusion is that the ^{99m}TcN-MoAb
35 complexes produced in this way were either unstable or
36 "sticky" and on exposure to target cells the ^{99m}TcN bound
37 nonspecifically.

38 $^{99m}TcNCl_4^-$ complexed to sulfhydryl groups after partial

1 reduction: As the former labelling method gave low
2 specificity (previously due to non-specific labelling) an
3 alternative method of labeling was designed, utilizing the
4 known ability of $^{99m}TcNCl_4^-$ to form a stable covalent
5 linkage to sulfur atoms. MoAb were partially reduced with
6 DTT to generate free sulfhydryl sites and mixed with the
7 $^{99m}TcNCl_4^-$, leading to the formation of ^{99m}TcN-MoAb. These
8 complexes were shown by sodium dodecyl sulfate
9 polyacrylamide gel electrophoresis (SDS-PAGE) to consist of
10 intact IgG. The binding assay demonstrated ^{99m}TcN-MoAb
11 complexes produced in this way to be specific and to yield
12 workable specificity ratios. The E3 (Ly-3+) cell line bound
13 8-10 times more ^{99m}TcN-anti-Ly-3 than did the BW5147 (Ly-3^-)
14 cell line (Figure 8a). Similarly two different MoAb, one
15 directed against colonic secretary epithelium (250-30.6) and
16 the other directed against Ly-2.1, were labeled with
17 $^{99m}TcNCl_4^-$ under identical conditions and the complexes were
18 tested for their ability to bind to the murine thymoma E3
19 (Ly-2.1^+, Ca Colon Ab^-) (Figure 8b), when the reactive MoAb
20 complex bound 6-8 times more efficiently than did the
21 ^{99m}TcN-anti-colon complex. In separate experiments the
22 ^{99m}TcN-colon MoAb, unable to bind to the E3 thymoma could
23 bind reactive human tumor cell lines, Colo 397 and Colo 205
24 incorporating 90-250 times more radioactivity than
25 nonreactive control cells (Figure 9).
26 The partial reduction method was then used to
27 radiolabel a panel of eleven different MoAb, including some
28 of the same specificity but of different isotypes. All MoAb
29 were tested in the binding assay, and bound specifically to
30 reactive target cells (Table 2). Four different Ly-2.1 MoAb
31 were tested; there was a 10-15 fold difference between the
32 binding of ^{99m}TcN-labeled IgG_{2a} and IgM anti-Ly-2.1
33 (monomer) MoAb on reactive target cells (CBA, RF/J) compared
34 to that found with nonreactive target cells (BALB/c,
35 C57BL/6), whereas the IgG_1 and IgG_3 Ly-2.1 MoAb produced
36 specificity ratios of 30-50 and 50-70 respectively. Other
37 MoAb were also highly selective e.g. anti-Ly-1.1 (IgG_{2a}) and
38 H129-19 (IgG_1) produced specificity ratios of 70-130 and

1 110-130 respectively (Table 2).

2 Radiolabeling of IgM MoAb (Pentamers) with $^{99m}TcNCl_4^-$:
3 In contrast to the preceding results obtained by direct
4 labeling (using amino groups) the direct complexing of
5 $^{99m}TcNCl_4^-$ via amino groups with IgM class of MoAb produced
6 ^{99m}TcN-MoAb complexes that gave 10 times more specific
7 binding than similar complexes made with IgG MoAb.
8 $^{99m}TcNCl_4^-$ directly complexed with anti-Thy-1.2 (IgM) MoAb
9 via amino groups produced specificity ratios of 15-20 (cpm
10 bound CBA / cpm bound AKR) when tested on thymocytes from
11 CBA (Thy-1.2$^+$) and AKR (Thy-1.1$^+$) strains (Figure 10).
12 However the partial reduction procedure, complexing
13 $^{99m}TcNCl_4^-$ through sulfur atoms, resulted in superior
14 specificity ratios, and CBA thymocytes (Thy-1.2$^+$)
15 incorporated more than 90 times more ^{99m}TcN-anti-Thy-1.2
16 than nonreactive AKR thymocytes (Thy-1.1$^+$). The
17 immunoperoxidase method was also used to assess the MoAb
18 activity of two IgM MoAb, before and after labeling with
19 $^{99m}TcNCl_4^-$: (a) 3E1.2, which reacts strongly with membrane
20 and cytoplasm of breast carcinoma and with the luminal
21 membrane of normal breast and (b) 5C-1, which reacts with
22 colonic carcinoma; the labeling procedure used did not
23 significantly alter the binding ability of the radiolabeled
24 MoAbs (Table 3).

25 Immunoreactivity of $^{99m}TcNCl_4^-$ labeled MoAb: It was
26 necessary to show that the partial reduction procedure used
27 to label MoAb with $^{99m}TcNCl_4^-$ did not significantly
28 compromise the binding ability of the MoAb to bind reactive
29 target cells and this was demonstrated in three ways. First
30 it was important to assess the immunoreactivity of the MoAb
31 retained after labeling and so the percentage of binding of
32 the radiolabeled ^{99m}TcN-MoAb was determined. ^{99m}TcN-anti-Ly-
33 2.1 was added to an increasing number of E3 cells (Ly-2$^+$),
34 and the amount of MoAb binding to the cells was determined
35 (Figure 11). The degree of nonspecific binding was
36 determined by running in parallel a nonreactive ^{99m}TcN-anti-
37 Ly-1.1 isotype control labeled under identical conditions.
38 The reactive ^{99m}TcN-anti-Ly-2.1 achieved significantly

1 higher binding to E3 cells (60% in the plateau region) than
2 the control ^{99m}TcN-anti-Ly-1.1 (5%) (not shown). Secondly
3 the binding of unmodified MoAb and ^{99m}TcN-labeled MoAb was
4 determined using the binding assay, where the amount of MoAb
5 bound to reactive cells was measured using a second antibody
6 (anti-immunoglobulin) which was iodinated and reactive with
7 the first. Increasing concentrations of unmodified anti-Ly-
8 2.1, DTT treated anti-Ly-2.1, ^{99m}TcN-anti-Ly-2.1 labeled to
9 a high (100 muCi/mug) or low specific activity (12 muCi/mug)
10 had equal binding capacity on E3 cells (Figure 12a), which
11 suggests that this technique had not impaired the binding
12 activity of the MoAb. This was also confirmed by the
13 rosetting assay, when the initial MoAb titer of 1:16,384 was
14 unaltered after radiolabeling (Figure 12b).

15 <u>Binding of ^{99m}TcN-anti-Ly-2.1 to tumor cell lines with</u>
16 <u>different concentrations of Ly-2.1:</u> To clarify that the
17 binding of the ^{99m}TcN-MoAb complexes was primarily dependent
18 on the concentration of reactive antigen binding sites on
19 the target cells, the binding assay was used, with 3
20 different tumor cell lines E3, D1 and BW5147, that differed
21 in concentrations of Ly-2 present on the cell surface. E3
22 and D1 are high and low Ly-2^+ variants of the ITT(1)75NS
23 cell line, and BW5147 being Ly-2^- was used as a control. The
24 amount of binding was in proportion to the antigen density
25 and ^{99m}TcN-anti-Ly-2.1 bound the E3 cell line 8 times more
26 antibody than the D1 cell line and incorporated up to 100
27 times more radiolabel than did the nonreactive BW5147 cell
28 line (Figure 13).

29 <u>Imaging:</u> The four different Ly-2.1 MoAb (IgG_1, IgG_{2a},
30 IgG_3 and IgM) were used in imaging experiments using B6CF1
31 mice bearing E3 tumor grafts to determine which subclass
32 best localized the tumor in vivo. In the first experiment
33 mice with E3 tumor (0.82 cm in diameter) located on one
34 thigh were given intravenous injections of ^{99m}TcN-anti-Ly-
35 2.1 (IgG_{2a}) or ^{99m}TcN-anti-Ly-1.1 (IgG_{2a}), the control
36 antibody. Scintigrams images obtained 28 hours after
37 injection demonstrated the specific localization of the
38 ^{99m}TcN-anti-Ly-2.1. Radioactivity was concentrated in the

1 central region of the mouse, indicative of the significant
2 antibody distribution to vascularized organs such as the
3 liver, lung and heart but the tumor was easily defined. When
4 ^{99m}TcN-anti-Ly-1.1 was used as a nonreactive isotype
5 control, the definition of the tumor was poor relative to
6 the images obtained with specific MoAb and only blood pool
7 activity in the tumor was observed, with no specific
8 localization. In another experiment B6CF1 mice hosting three
9 E3 tumors were scanned 28 hours after the intravenous
10 administration of ^{99m}TcN-anti-Ly-2.1 (IgG_{2a}), and all three
11 tumors could be visualized. However the high blood pool
12 activity hindered visualization of the tumor close to the
13 vascular organs such as the heart and liver.
14 The IgM Ly-2.1 MoAb (monomer) was used to specifically
15 localized E3 tumors in B6CF1 mice, and mice with two E3
16 tumors (0.62 cm and 0.65 cm in diameter) were scanned 4 and
17 28 hours after an intravenous injection. Both tumors could
18 be visualized 4 hours after injection, and the tumors became
19 progressively better defined with time. From the scans
20 obtained it was apparent that the IgG_{2a} Ly-2.1 MoAb resulted
21 in superior images compared to the images obtained with the
22 IgM MoAb and also those obtained with the IgG_1 and IgG_3 MoAb
23 (data not shown).
24 DISCUSSION
25 The use of ^{99m}Tc as a radiolabel for
26 immunoiscintigraphy with MoAbs has been advocated as it is
27 one of the most useful radionuclides because of its ideal
28 nuclear properties (T1/2=6 hr, energy 140keV, with no beta
29 emmission). However little use has been made of this
30 radionuclide for radiolabeling MoAb because of the complex
31 chemistry involved in satisfactorily attaching it to
32 antibody. Several different methods have been used to
33 complex ^{99m}Tc to MoAb. The first relied on the conjugation
34 of the metallic radionuclide via bifunctional chelates, of
35 proven success for ^{111}In in both experimental (5-8) and
36 clinical application (9-11) but of little value for ^{99m}Tc.
37 The second approach involved the direct complexing of ^{99m}Tc
38 to MoAb to produce complexes with either amino groups or

1 sulfhydryl groups, the latter shown to form stable complexes
2 in vitro (14,15). However, problems are associated with the
3 methods used to reduce ^{99m}Tc prior to coupling with MoAb,
4 such as the production of colloid and the instability of the
5 ^{99m}Tc-labeled MoAb complexes (16,19).
6 We now describe a simple one step method of
7 radiolabeling MoAb with $^{99m}TcNCl_4^-$ based on a substitution
8 reaction and have complexed $^{99m}TcNCl_4^-$ to MoAb via two
9 different ligands, either the amino groups or sulfhydryl
10 groups and during our studies we observed that the two
11 complexes behaved differently. The complexes produced by
12 reacting $^{99m}TcNCl_4^-$ directly with amino groups of MoAb was
13 characterized by low specificity ratios as the ^{99m}TcN bound
14 equally well to reactive and nonreactive target cells
15 (Figure 7). The complexes formed with the use of sulfyhydryl
16 groups are clearly different from those formed with the use
17 of amino groups and ligands that bind through sulfur atoms
18 produce more stable complexes. To utilize the known
19 preference of the TcN group for sulfur atoms we developed a
20 partial reduction procedure used for the conversion of
21 disulfide linkages to sulfhydryl residues. Such an approach
22 was also attractive because it was known that the sulfhydryl
23 groups involved in the coupling were likely to be distant
24 from the sites responsible for antibody binding.
25 Important features of our study here show that the
26 coupling procedure used to attach $^{99m}TcNCl_4^-$ to MoAb is
27 simple, efficient and reliable and can be applied to a
28 number of MoAbs of either IgG or IgM classes. There is no
29 need for long incubation times required with the pretinning
30 method necessary for the reduction of ^{99m}Tc (16). The
31 labeling efficiency of ^{99m}TcN-labeled MoAb ranged between
32 80-90% (Figure 6) and results obtained from TCA protein
33 precipitation determinations of radiolabeled MoAb indicated
34 that up to 98-99% of the ^{99m}TcN is bound. The labeling
35 procedure adopted did not damage the binding specificity of
36 the antibody molecule and did not alter the antibody antigen
37 binding capacity (Figure 12) as can occur with the
38 conjugation of metallic radionuclides via bifunctional

1 chelates (6). Up to 60% of the radiolabeled preparation was
2 able to bind specifically to target cell (Figure 11).
3 Furthermore, the degree of antibody binding was dependent on
4 the antigen density of the target cells, hence the Ly-2HIGH
5 E3 cell line bound 8 times more ^{99m}TcN-anti-Ly-2.1 than the
6 Ly-2LOW D1 cell line (one has approximately 8 times the
7 antigen density of the other) and 10 times more than the
8 nonreactive BW5147 (Ly-2.1^{-}) cell line (Figure 13). Finally,
9 high specific activities were achieved which allowed the
10 specific localization of labeled MoAb in the appropriately
11 reactive murine tumors.
12 Specific localization by immunoscintigraphy of murine
13 tumors was demonstrated with ^{99m}TcN-labeled MoAb by imaging
14 studies and was illustrated in two ways. First, imaging
15 studies showed that the E3 (Ly-2.1^{+}) tumors were visible
16 when mice were injected with reactive ^{99m}TcN-anti-Ly-2.1
17 whereas identical tumors could not be localized with
18 nonreactive ^{99m}TcN-anti-Ly-1.1 an isotype identical control.
19 Second ^{99m}TcN-MoAb could specifically detect more than one
20 tumor and this study showed that several tumors in the one
21 mouse could be specifically localized. The method is useful
22 to detect murine tumors and results indicate value in
23 patients with cancer.

TABLE 1

Characteristics of murine monoclonal antibodies used for radiolabeling with $^{99m}TcNCl_4^-$

Antibody (Ref)	Antigen Specificity	Antibody Class and Subclass	Antibody purification
anti-Ly-2.1(21)	Ly-2.1	IgG_{2a}	Protein A
		IgG_1	Protein A
		IgG_3	Protein A
		IgM(monomer)	DEAE
anti-Ly-3.1(22)	Ly-3.1	IgG_1	DEAE
anti-Ly-1.1(23)	Ly-1.1	IgG_{2a}	Protein A
anti-Ly-15.2(24)	Ly-15.2	IgG_{2a}	DEAE
anti-Thy-1.2	Thy-1.2	IgM(pentamer)	$NH_4(SO_4)_2$
H129-19(25)	L3T4	IgG_{2a}	DEAE
HuLy-m9	human transferrin receptor	IgG_1	Protein A
250-30.6(26)	colon	IgG_{2b}	Protein A
3E1.2(27)	breast	IgM(pentamer)	precipitation in water
5C-1(28)	colon	IgM(pentamer)	precipitation in water

TABLE 2

Specificity ratios of ^{99m}TcN-labeled MoAb, prepared by the partial reduction of MoAb with DTT and obtained from in vitro binding studies.

Antibody Designation	Origin of Target cells: Reactive	Origin of Target cells: Nonreactive	Specific Ratio[1]
anti-Ly-2.1			
IgM(monomer)	CBA	BALB/c	10-15
IgG_3	CBA	BALB/c	50-70
IgG_1	CBA	BALB/c	30-50
IgG_{2a}	RF/J	C57BL/6	10-15
anti-Ly-3.1	E3 thymoma	BW5147	7-11
anti-Ly-1.1	CBA	BALB/c	70-130
anti-Ly-15.2	RF/J	BALB/c	50-60
anti-Thy-1.2	CBA	AKR	60-90
HuLy-m9	CEM	E3	120-200
250-30.6	COLO 205	BORDIN (EBV)	150-250
	COLO 397	BORDIN (EBV)	90-180
H129-19	E3 thymoma	BW5147	110-130

1 Specific ratio = cpm bound reactive cells / cpm bound nonreactive cells.

TABLE 3

Effect of conjugation with $^{99m}TcNCl_4^-$ on the reactivity of IgM MoAb.

A. Reaction of MoAb 3E1.2 with carcinoma of the breast.1

Antibody Dilution	Treatment			
	none	DTT	^{99m}TcN-labeled	
			-NH2	-SH
10-3	++++	++++	++++	++++
10-4	+++	+++	+++	++
10-5	++	++/+	++	+
10-6	+	+/-	+/-	+/-

B. Reaction of MoAb 5C-1 with normal colon.1

Antibody Dilution	Treatment			
	none	DTT	^{99m}TcN-labeled	
			-NH2	-SH
10-2	++++	++++	++++	++++
10-3	+++	+++	+++	++
10-4	+	+	+	+

1 Tissues were tested by immunoperoxidase and specificity graded: 0 = negative, + = weak, ++ = moderate, +++ = strong, ++++ = very strong.

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FIGURE LEGENDS

Figure 6 Purification of $^{99m}TcNCl_4^-$-labeled MoAb using Sephadex G25. The column was equilibrated with PBS and 0.5 ml fractions were collected.

Figure 7 Binding of $^{99m}TcNCl_4^-$-labeled anti-Ly-2.1 prepared by direct labeling of MoAb on RF/J (●) and BIO (o) thymocytes (Fig. 7a) or anti-Ly-2.1 (●) and anti Ca. colon (o) on E3 thymoma cells (Fig. 7b). The amount of radioactivity bound as a function of antibody concentration is shown.

Figure 8 Specific binding to E3 target cells (8a)

1 anti-Ly-3 labeled with $^{99m}TcNCl_4^-$ to DTT treated E3 (●) and
2 BW5147 (O) target cells; or (8b) anti-Ly-2.1 (●) and anti-
3 colon antibody (O).
4 Figure 9 Specific binding of DTT treated anti-colon
5 antibody labeled with $^{99m}TcNCl_4^-$ to Colo 397 (●), Colo 205
6 (O), and a non-reactive control (EBV derived tumour) (▲).
7 Figure 10 Binding of 2 different anti-Thy-1.2
8 conjugates - one prepared with direct labeling of MoAb with
9 $^{99m}TcNCl_4^-$ (—) and the other prepared with DTT treated MoAb
10 (- - - -) or CBA (●) and AKR (O) thymocytes.
11 Figure 11 Percentage binding of $^{99m}TcNCl_4^-$-labeled
12 anti-Ly-2.1 (●) to E3 target cells; the control anti-Ly-1.1
13 antibody did not bind >5% at any dilution (not shown). The
14 amount of radioactivity incorporated as a function of cell
15 number is shown.
16 Figure 12 Binding of anti-Ly-2 to E3 target cells,
17 using unmodified anti-Ly-2.1 (▲); DTT treated anti-Ly-2.1
18 (o), $^{99m}TcNCl_4^-$ labeled anti-Ly-2.1, high specific activity
19 (100muCi/mug) (●) and low specific activity (12muCi/mug)
20 (■). (12a) detection using ^{125}I-sheep anti-mouse Ig; (12b)
21 rosetting using sheep anti-mouse Ig.
22 Figure 13 Binding of DTT treated anti-Ly-2.1 labeled
23 with $^{99m}TcNCl_4^-$ to E3 (●), D1 (Δ) and BW5147 (o) target
24 cells. The amount of radioactivity as a function of antibody
25 concentration is shown.

1 The claims defining the invention are as follows:
2 1. A conjugate of technetium with a radical having an
3 antigen binding site wherein the technetium thereof is
4 radioactive.
5 2. A conjugate as claimed in claim 1, wherein the
6 technetium is ^{99m}Tc.
7 3. A conjugate as claimed in claim 1 or claim 2, wherein
8 said radical is an antibody or antibody fragment which is
9 preferentially absorbed by a tumour cell as compared to a
10 non-tumour cell.
11 4. A conjugate as claimed in any preceding claim, wherein
12 the conjugation is via a sulphide linkage.
13 5. A conjugate as claimed in any preceding claim, and of
14 formula I
15 $Ab\text{-}Y\text{-}S\text{-}NTc(Hal)_3$ Formula I
16 wherein Hal is chlorine, bromine or iodine and including
17 mixed halides, Ab is a radical having an antigen binding
18 site and Y is a conjugating chain.
19 6. A conjugate as claimed in claim 5, wherein Y is of
20 formula II
21 X Z
22 " ' Formula II
23 $-[NH\text{-}C\text{-}(CH)_n]_z-$
24 wherein Z is H, alkyl, aryl, carboxy, halide, hydroxy or
25 amino, X is NH, O or S and z is 0 or 1.
26 7. A conjugate as claimed in claim 5 and of formula
27 $Ab\text{-}S\text{-}NTc(Hal)_3$ wherein Ab and Hal have the meaning given in
28 claim 5 or Ab-S represents an antibody radical or a radical
29 having an antigen binding site.
30 8. A conjugate as claimed in claim 5 and of formula
31 $Ab\text{-}NH\text{-}Y\text{-}S\text{-}NTc(Hal)_3$ wherein Ab and Ab-NH represent an
32 antibody radical or a radical having an antigen binding site
33 and Y and Hal have the meaning given in claim 5.
34 9. A conjugate as claimed in any preceding claim, wherein
35 said radical is an antibody, an antibody polymer, an
36 antibody monomer or an antibody fragment having an antigen
37 binding site.
38 10. A conjugate as claimed in any preceding claim, wherein

1 said radical is an antibody, an antibody polymer, an
2 antibody monomer or an antibody fragment having an antigen
3 binding site selected from the group showing specificity for
4 one of breast, brain, melanoma, lung, pancreas and colon
5 tumours.
6 11. A conjugate as claimed in claim 9, wherein said radical
7 is an antibody fragment having an antigen binding site and
8 selected from $F(ab')_2$, F(ab'), IgG_1, IgG_{2a}, IgG_{2b} and IgG_3.
9 12. A pharmaceutical composition comprising a compound in
10 accordance with any preceding claim together with a
11 pharmaceutically acceptable diluent.
12 13. A compound of formula
13 Ab-SH or
14 Ab-NH-Y-SH
15 wherein Ab, Ab-NH, Ab-S and Y have the meaning given in
16 claims 7 and 8.
17 14. A method of making a conjugate in accordance with any
18 one of claims 1 - 11 comprising taking a compound in
19 accordance with claim 13 and reacting it with $TcN(Hal)_4^-$
20 wherein Hal is chlorine, bromine or iodine and including
21 mixed halides.
22 15. A method as claimed in claim 14, including obtaining
23 the compound of claim 13 by reducing an antibody or a
24 compound having an antigen binding site to form free
25 sulphydryl groups.
26 16. A method as claimed in claim 14, including obtaining
27 the compound of claim 13 by reacting an antibody or compound
28 having an antigen binding site succinimidyl
29 pyridyldithiopropionate (SPDP) or an analogue thereto
30 appropriate to the compound of claim 13 desired to obtain an
31 antibody or compound having an antigen binding site
32 conjugate containing a -S-S- group and reducing the
33 conjugate to form a -SH group.
34 17. A method as claimed in claim 14, including obtaining
35 the compound of claim 13 by using S-acetylmercaptosuccinic
36 anhydride (SAMSA) or SH introducing compounds to produce a
37 side chain on an antibody or compound having an antigen
38 binding site containing a -S- linkage and reducing to form a

1 -SH group.
2 18. A conjugate or method of making same substantially as
3 hereinbefore described with reference to any one of the
4 preparations.
5 19. The articles, things, parts, elements, steps, features,
6 methods, processes, compounds and compositions referred to
7 or indicated in the specification and/or claims of the
8 application individually or collectively, and any and all
9 combinations of any two or more of such.

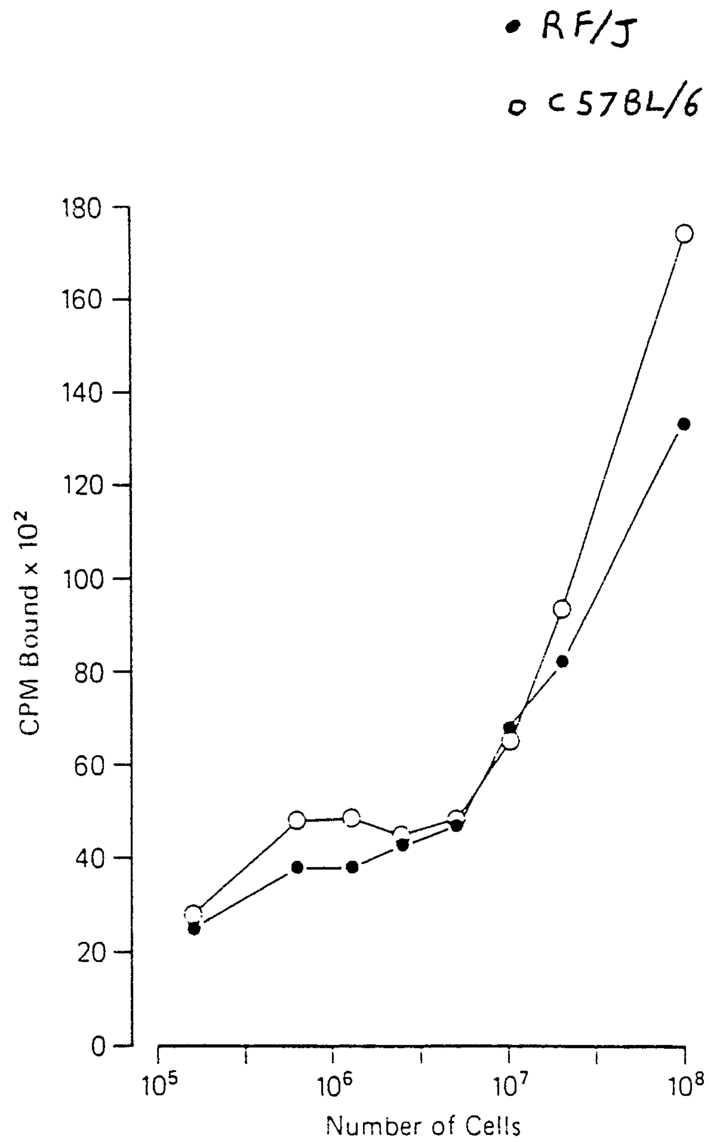


Fig. 1

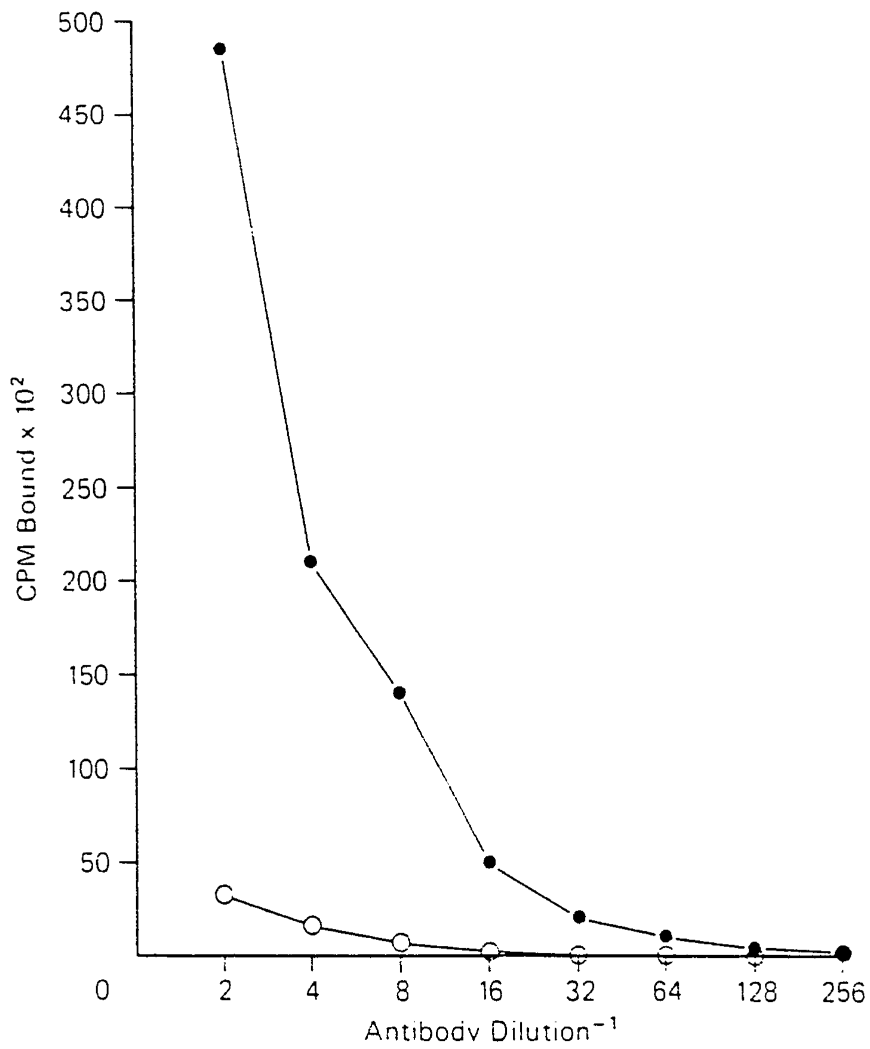


Fig. 2

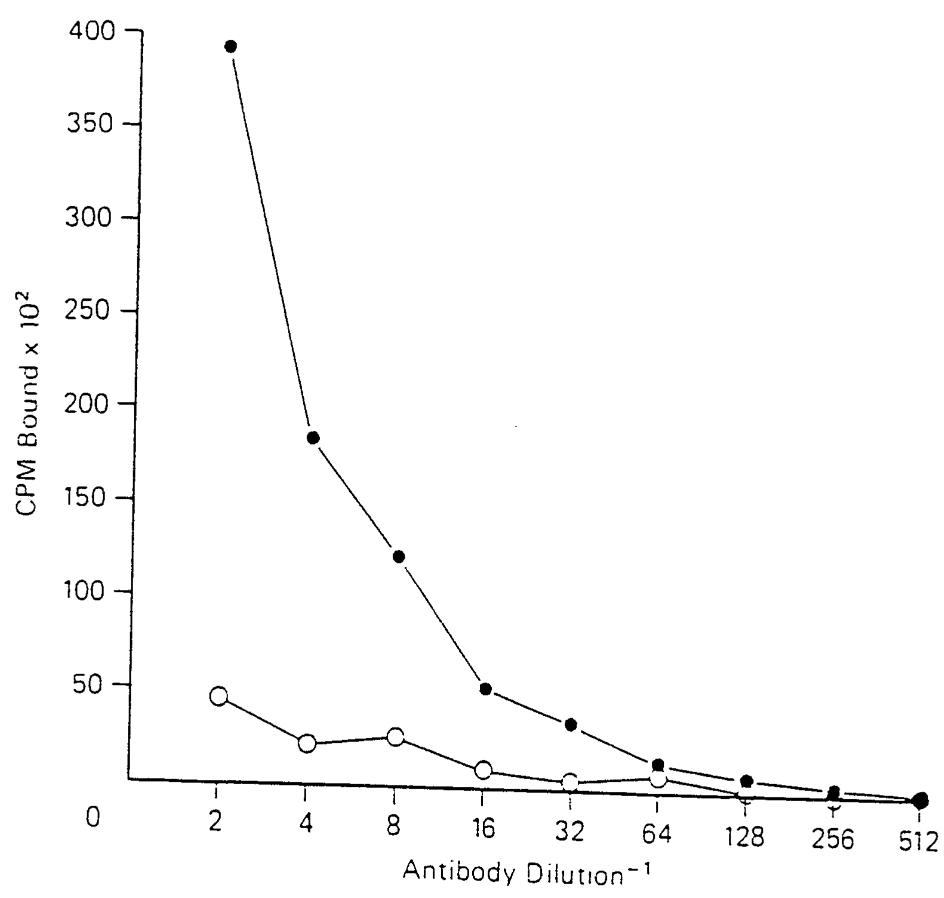


Fig. 3

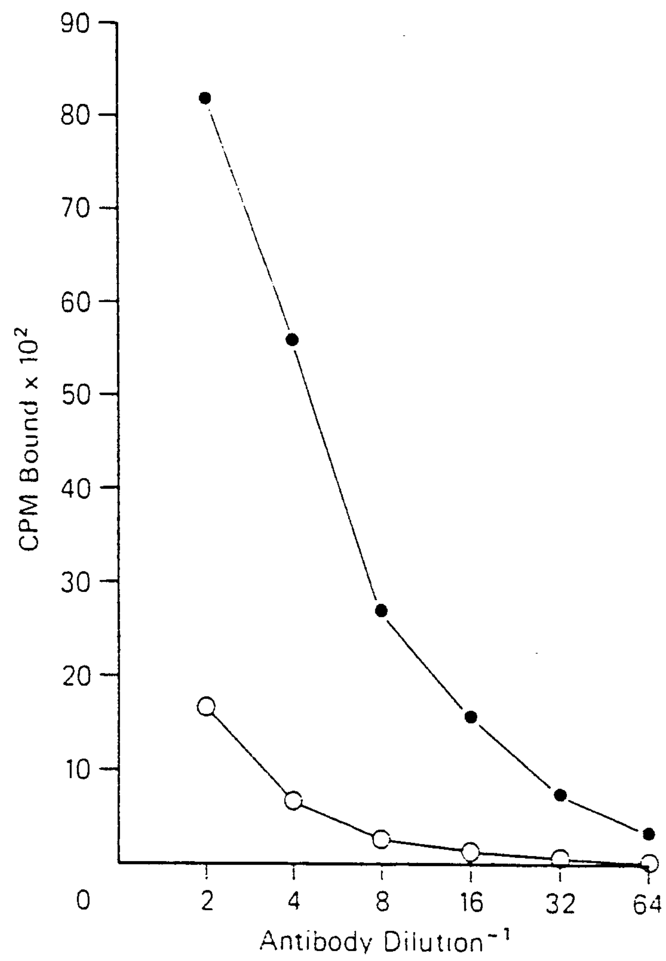


Fig.4

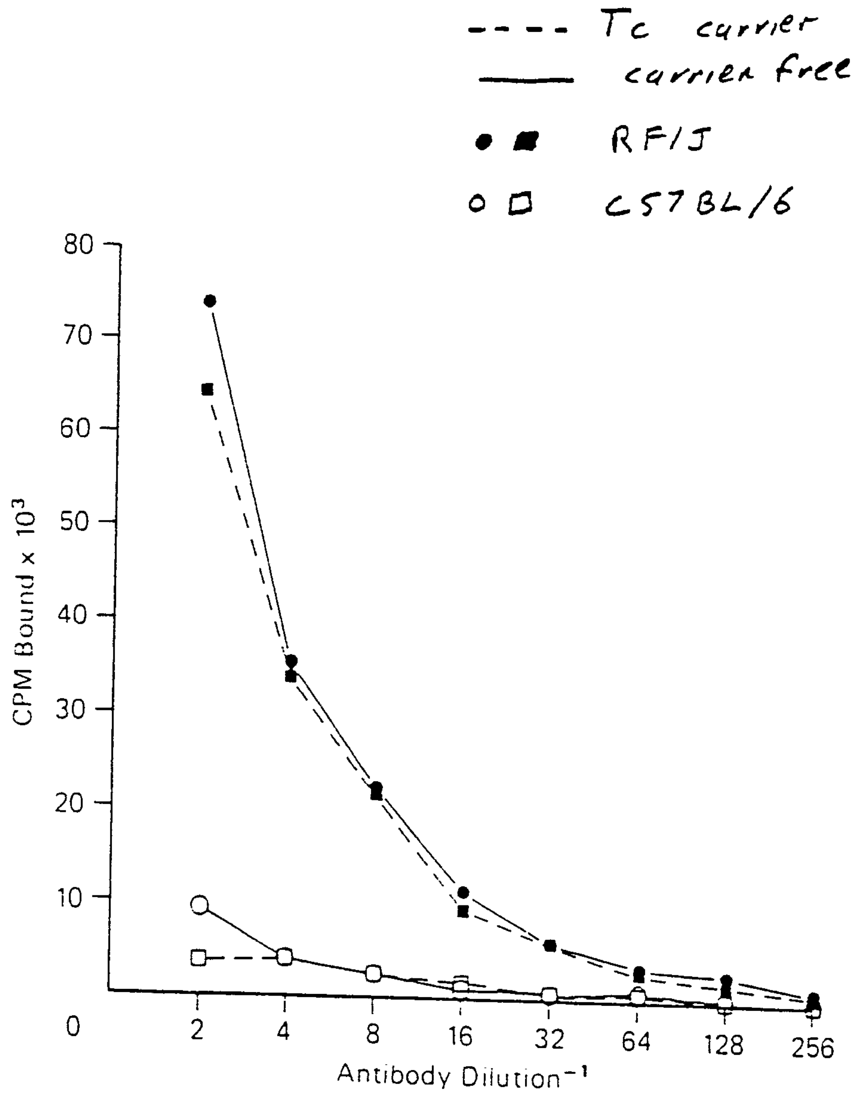


Fig. 5

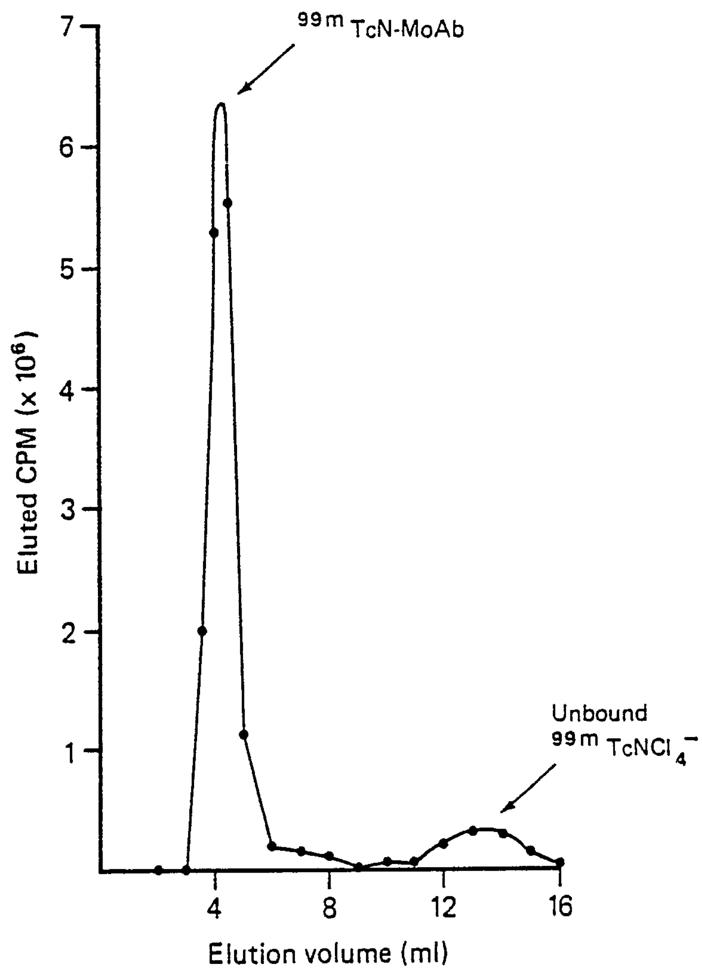


Fig. 6

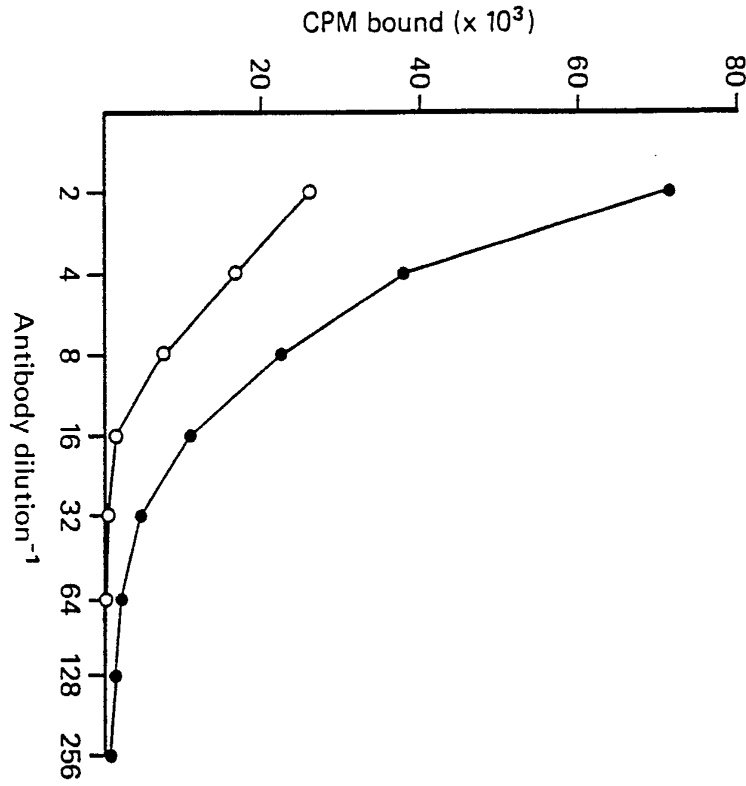


Fig. 7b

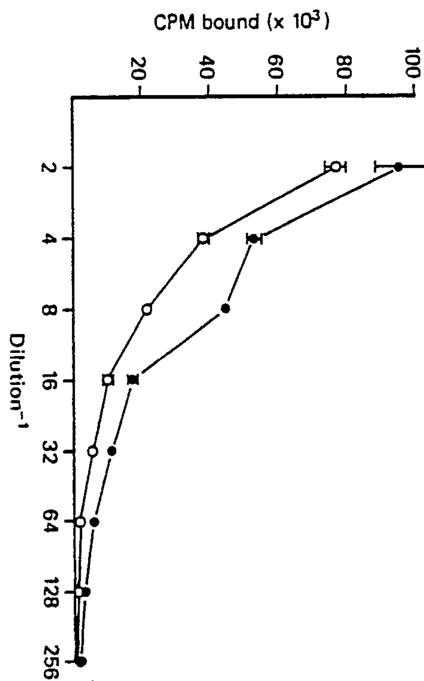


Fig. 7a

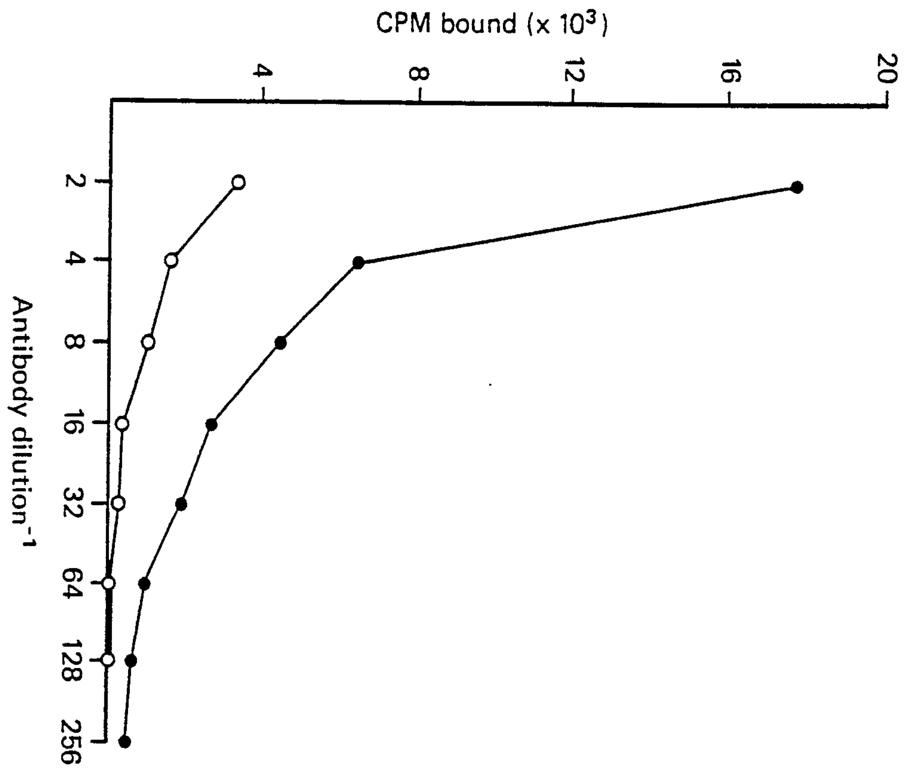


Fig. 8a

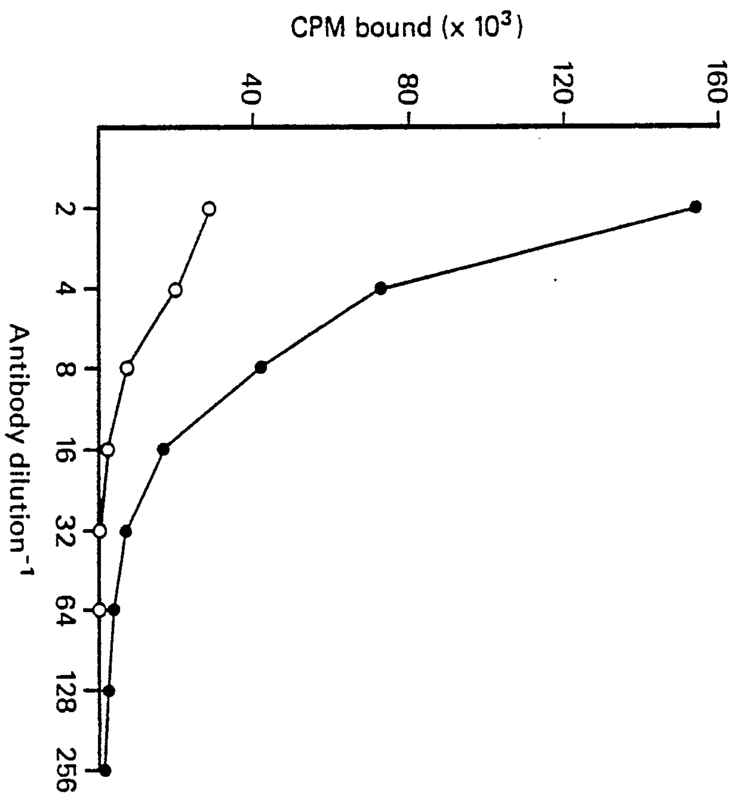


Fig. 8b

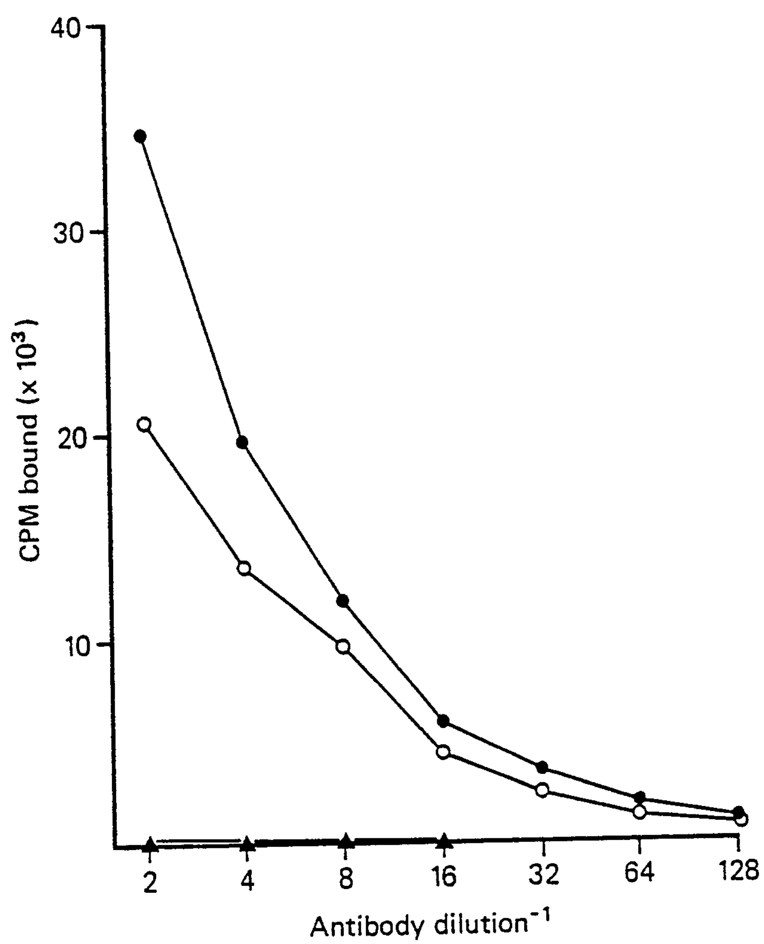


Fig. 9

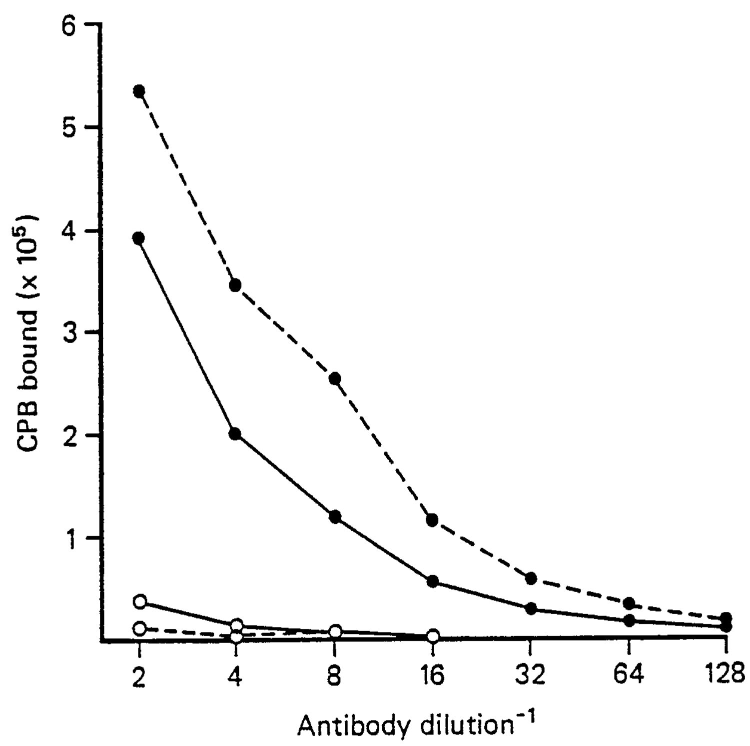


Fig. 10

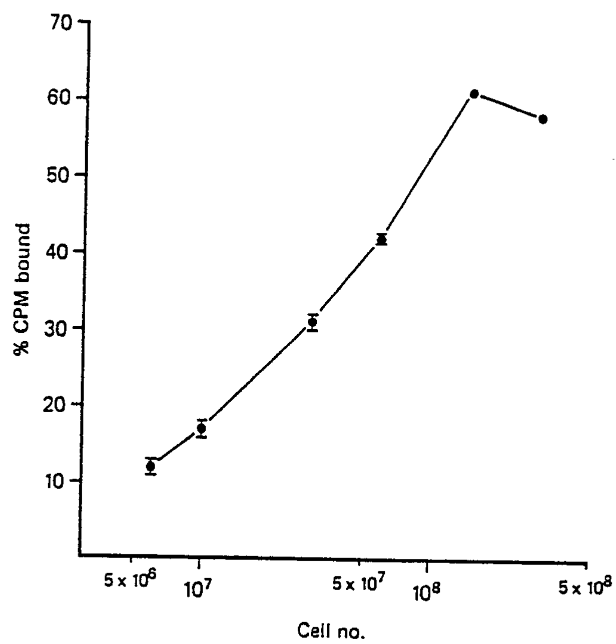


Fig. 11

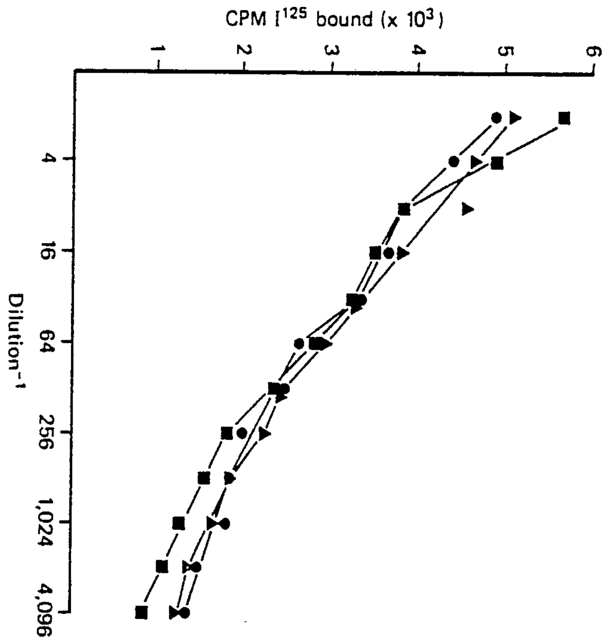


Fig.12a

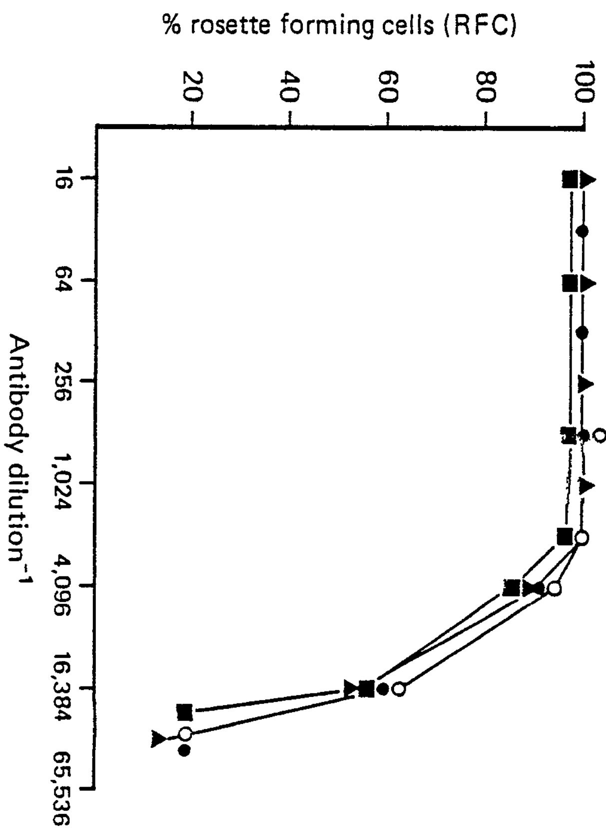


Fig.12b

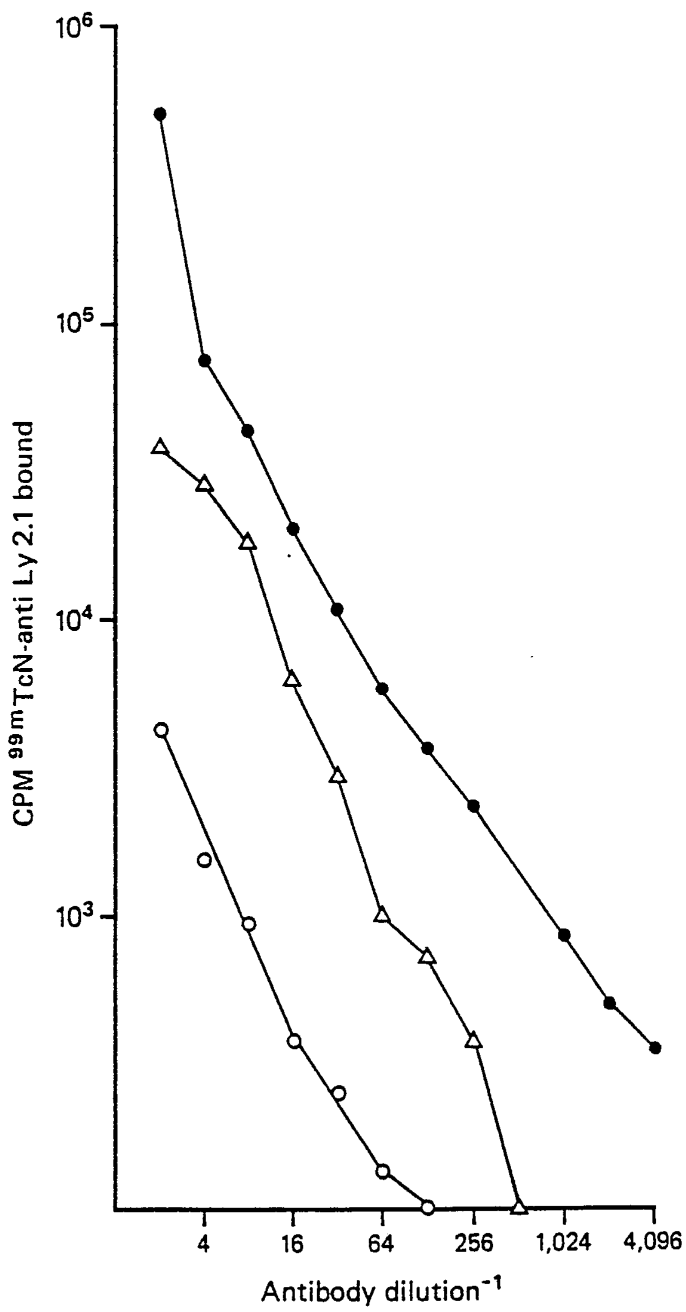
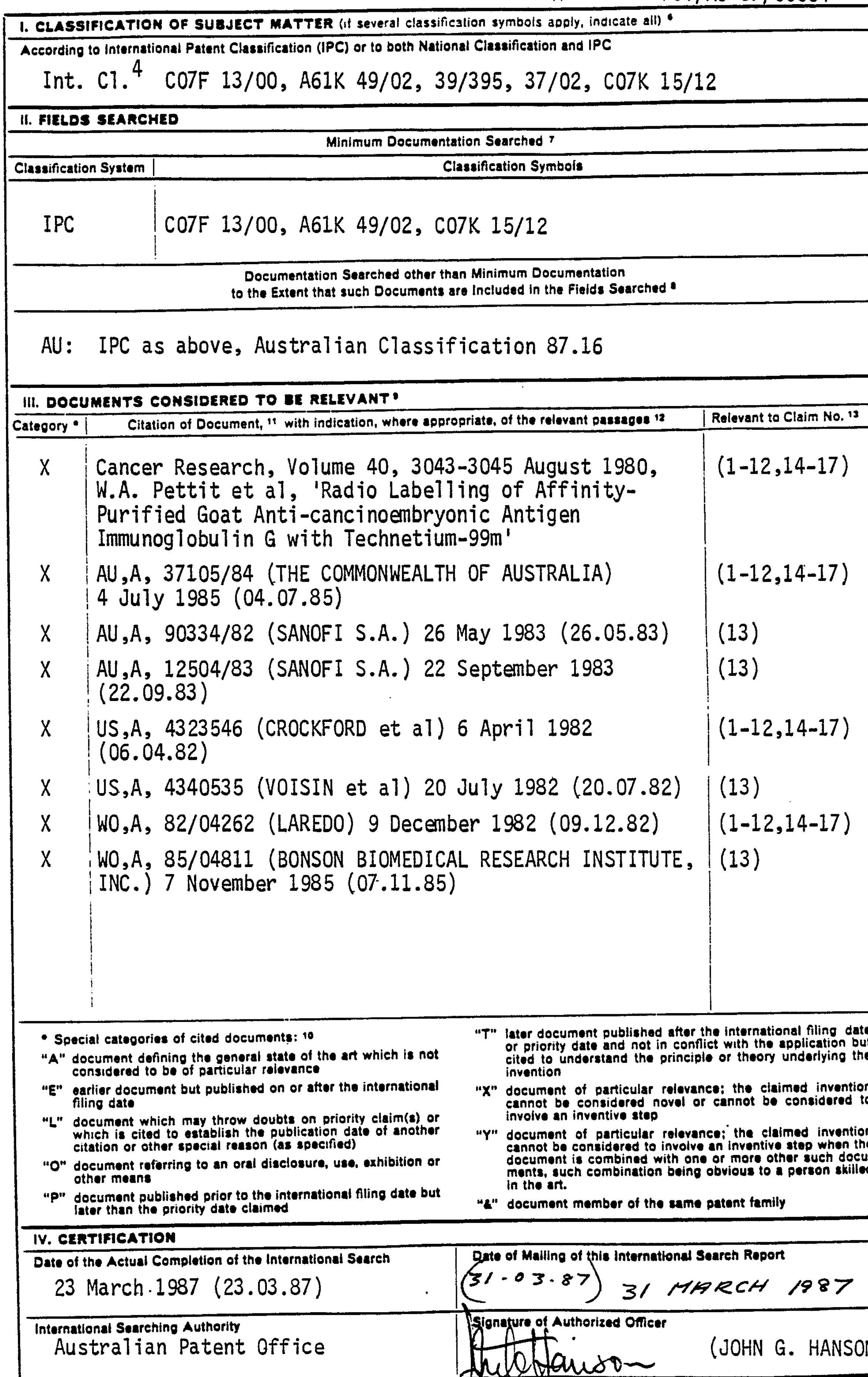


Fig. 13



INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00004

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07F 13/00, A61K 49/02, 39/395, 37/02, C07K 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07F 13/00, A61K 49/02, C07K 15/12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC as above, Australian Classification 87.16

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Cancer Research, Volume 40, 3043-3045 August 1980, W.A. Pettit et al, 'Radio Labelling of Affinity-Purified Goat Anti-cancinoembryonic Antigen Immunoglobulin G with Technetium-99m'	(1-12,14-17)
X	AU,A, 37105/84 (THE COMMONWEALTH OF AUSTRALIA) 4 July 1985 (04.07.85)	(1-12,14-17)
X	AU,A, 90334/82 (SANOFI S.A.) 26 May 1983 (26.05.83)	(13)
X	AU,A, 12504/83 (SANOFI S.A.) 22 September 1983 (22.09.83)	(13)
X	US,A, 4323546 (CROCKFORD et al) 6 April 1982 (06.04.82)	(1-12,14-17)
X	US,A, 4340535 (VOISIN et al) 20 July 1982 (20.07.82)	(13)
X	WO,A, 82/04262 (LAREDO) 9 December 1982 (09.12.82)	(1-12,14-17)
X	WO,A, 85/04811 (BONSON BIOMEDICAL RESEARCH INSTITUTE, INC.) 7 November 1985 (07.11.85)	(13)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 March 1987 (23.03.87)	(31-03-87) 31 MARCH 1987
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	(JOHN G. HANSON)

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/AU 87/00004

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers..........., because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claim numbers..........., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers.........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(a) Claims 1 to 12 and 14 to 17 - Conjugates of Technetium.

(b) Claim 13 - Compounds containing an antibody.

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/AU 87/00004

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 37105/84	EP 166746	WO 8503063	
AU 90334/82	CA 1209472	EP 80401	FR 2516794
	JP 58135821	NZ 202563	US 4643895
	ZA 8208048		
AU 12504/83	CA 1216791	DD 209578	DK 1216/83
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US 4323546	CA 1116081	DK 2073/79	EP 5638
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WO 8204262	AU 84589/82	CA 1215919	DK 519/83
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WO 8504811	AU 42901/85	DK 6019/85	DK 6019/85
	EP 179872	FI 855086	NO 855200

END OF ANNEX