A New Radioisotopic Microassay of Cell-Mediated Immunity Utilizing Technetium-99m Labeled Target Cells[1] (37026)

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Previously described *in vitro* radioisotopic assays of lymphocyte or antibody-mediated cytotoxicity employed nuclides or compounds which could be degraded, released and/or reutilized during the course of an experiment (1–14). Such release and reutilization of isotope could be a serious disadvantage in tests where target cells and lymphocytes were admixed with one another for intervals of time greater than a few hours.

We now describe a new radioisotopic microcytotoxicity assay employing technetium-99m (^{99m}Tc), a nuclide which is bound by reduction to cellular components and does not appear to be released in a reutilizable form by dead or injured cells (15, 16). The development of immunity in C57Bl/6 mice (H-2^b) to Sarcoma I (H-2^a) tumor cells was assessed *in vitro* by means of the ^{99m}Tc assay and the data obtained have been compared with the tritiated thymidine (13) and ^{125}I-5-iodo-2′-deoxyuridine (^{125}I-IDU) isotopic assays (14), and with a microcytotoxicity assay based on the visual counting of surviving cells (17, 18).

Materials and Methods. Preparation of radiolabeled target cells. Technetium-99m is a metastable isotope ($T_{½}$ = 6.0 hr) emitting a gamma photon with an energy of 140 keV. Our preparations were obtained as sodium pertechnetate ($Na^{99m}TcO_4$) from a New England Nuclear molybdenum-99 generator eluted with 0.9% NaCl. Labeling was accomplished by adding 10 mCi of ^{99m}Tc to 10 ml of Hanks' balanced salt solution (HBSS) containing 10^7 Sarcoma I (Sa I) cells. After incubation for 30 min at 37° with occasional agitation 0.4 ml of a 0.1% solution of $SnCl_2 \cdot 2H_2O$ was added to reduce the ^{99m}Tc from a valence state of +7 to +2 (19). Following an additional 15 min incubation, the cell suspension was washed three times with HBSS to remove unbound isotope. The radiolabeled tumor cells were resuspended in HBSS, viability was determined by trypan blue exclusion (20), and the final concentration was adjusted to 10^6 living cells/ml.

Preparation of lymphocytes and sera. The alloimmune reactivity of C57Bl/6 (H-2^b) mice to Sa I (H-2^a) tumor target cells was determined 3, 7, 10, 17, and 21 days following a single subcutaneous inoculation of 0.1 cm^3 of minced tumor tissue (approx 20 × 10^6 cells). Normal nontumor bearing C57Bl/6 mice of the same age and sex served as controls. On each day of the assay two mice from each group were bled from the retro-orbital sinus and the sera were separated, heat-inactivated (56° for 30 min) and stored in an ice bath until use. The axillary, cervical, inguinal and mesenteric lymph nodes of these same animals were excised aseptically, and lymphocyte suspensions were prepared by mincing the nodes, suspending them in ice-cold HBSS, and passing them through progessively higher gauge needles attached to a 6 ml syringe. Viability of the lymphocyte preparations ranged from 88 to 98% as determined by trypan blue exclusion. Final lymphocyte concentration was adjusted to

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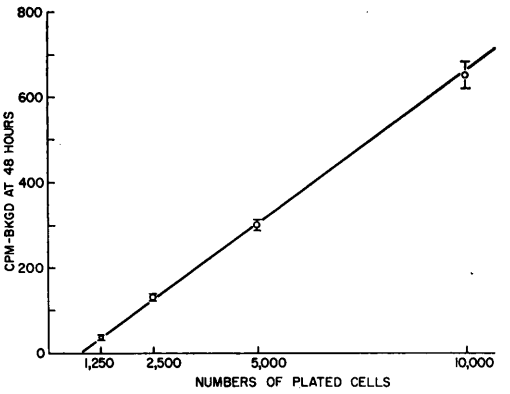

FIG. 1. Relationship between radioactivity expressed as cpm minus background (bkgd) and graded numbers of ^{99m}Tc labeled Sarcoma I cells alone following 48 hr incubation at 37° in Microtest II plates. Each point represents the mean of 8 to 16 replicate wells ± SE.

50 × 10^6 cells/ml in HBSS.

Technetium-99m microcytotoxicity assay. 10,000 viable ^{99m}Tc labeled Sa I cells were dispensed into each of 96 wells of a Falcon Microtest II plate (No. 3040) using a 500 μl Hamilton syringe with repeating dispenser. Each well of the plate contained 0.2 ml of Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM-10%NCS). Ten microliters of the lymphocyte suspension (5 × 10^5 cells) alone or in combination with 10 μl of test or control sera were added to six or eight replicate wells. Antibody-mediated complement dependent cytotoxicity was studied by adding 10 μl of serum together with a 1:4 dilution of pretested, noncytotoxic rabbit serum as a complement source. After 40 to 48 hr incubation at 37° in a humidified incubator containing 5% CO_2 and 95% air, the plates were gently washed twice with HBSS to remove dead and injured cells, air-dried and then spray-fixed with a surgical adhesive (Aeroplast). The bottoms of the wells were punched out using a length of metal tubing 6 mm in diameter and radioactivity was determined with a Nuclear-Chicago Model 1185 gamma scintillation counter.

Binding affinity of ^{99m}Tc. In order to determine maximum release of radioisotope triplicate samples of 10,000 labeled cells were added to 10 × 75 mm tubes containing 1.0 ml distilled water and subjected to 3 cycles of freeze-thawing to produce lysis. Following centrifugation (800*g* for 30 min) 0.5 ml of supernatant was removed and the radioactivity in the supernatant and sediment was determined. Less than 9% of the total bound isotope was found to be in the supernatant. The linear relationship between radioactivity expressed as cpm and graded numbers of Sa I cells after 48 hr in culture is presented in Fig.

TABLE I. The Development of Alloimmune Reactivity of C57Bl/6 Mice to Sarcoma I Following Tumor Challenge.

Test conditions[a]	Day:	Relative % target cell survival ± SE[b]				
		3	7	10	17	21
Control lymphocytes		92.9 ± 2.6	51.8 ± 2.4	84.0 ± 3.4	99.5 ± 10.7	62.5 ± 2.7
Test lymphocytes		92.5 ± 6.4	37.1 ± 6.1	26.0 ± 1.7	67.1 ± 4.0	73.5 ± 2.7
Control lymphs + test sera		57.7 ± 3.3	37.4 ± 4.4	59.1 ± 2.1	83.9 ± 3.0	56.9 ± 3.7
Test lymphs + test sera		68.8 ± 3.4	37.0 ± 2.8	23.9 ± 2.1	64.5 ± 5.3	62.2 ± 6.3
Control lymphs + control sera		57.6 ± 2.5	37.9 ± 3.8	71.9 ± 3.2	106.1 ± 4.4	43.2 ± 6.7
Test lymphs + control sera		72.1 ± 4.4	35.3 ± 2.5	29.2 ± 2.8	47.1 ± 4.7	46.2 ± 2.3
Control sera + complement		53.5 ± 1.3	82.9 ± 6.9	65.6 ± 4.7	61.1 ± 2.5	45.8 ± 1.7
Test sera + complement		58.9 ± 1.2	92.6 ± 5.2	67.8 ± 4.6	67.9 ± 4.6	58.3 ± 8.5

[a] Either control or test lymphocytes alone or in combination with sera were added to 10,000 ^{99m}Tc labeled target cells and then incubated for 40–48 hr at 37°. Control lymphocytes and sera were obtained from normal nontumor bearing C57Bl/6 mice.

[b] This is the ratio of the mean cpm of any control or test group compared to the mean cpm of 10,000 labeled target cells alone. Reactivity of test mice following subcutaneous inoculation of 20 × 10^6 SaI cells was determined on the days indicated.

TABLE II. Alloimmune Reactivity of C57Bl/6 Mice to Sarcoma I 10 Days Following Tumor Challenge.

Test conditions[a]	Relative % target cell survival ± SE[b]			
	^{99m}Tc	^{3}H	^{125}I	Visual
Control lymphocytes	84.0 ± 3.4	71.3 ± 7.7	73.9 ± 6.0	65.2 ± 4.8
Test lymphocytes	26.0 ± 1.7	11.2 ± 0.5	10.0 ± 0.3	65.7 ± 6.3
Control lymphs + test sera	59.1 ± 2.1	71.6 ± 5.1	46.4 ± 2.8	38.6 ± 3.4
Test lymphs + test sera	23.9 ± 2.1	14.3 ± 0.7	14.8 ± 1.0	13.5 ± 2.4
Control lymphs + control sera	71.9 ± 3.2	43.6 ± 1.7	55.4 ± 6.3	
Test lymphs + control sera	29.2 ± 2.8	25.2 ± 1.2	15.2 ± 1.1	
Control sera + complement	65.6 ± 4.7	35.8 ± 2.2	100.0 ± 7.5	75.4 ± 6.3
Test sera + complement	67.8 ± 4.6	45.3 ± 2.7	72.5 ± 8.9	61.8 ± 5.3

[a] Either control or test lymphocytes alone or in combination with sera were added to 10,000 ^{99m}Tc labeled target cells and then incubated for 40–48 hr at 37°. Control lymphocytes and sera were obtained from normal nontumor bearing C57Bl/6 mice.

[b] This is the ratio of the mean cpm of any control or test group compared to the mean cpm of 10,000 labeled target cells alone. Reactivity of test mice following subcutaneous inoculation of 20 × 10^6 SaI cells was determined by the technetium-99m (^{99m}Tc), tritiated thymidine (^{3}H) ^{125}I-iodo-deoxyuridine (^{125}I) isotopic assays and visual counting of surviving cells.

1. Although the actual number of surviving cells per test well could be determined by interpolation, it was more convenient to express the data as percentage of surviving cells. This is a ratio of the mean cpm of any control or test group compared with the mean cpm of 10,000 labeled target cells alone.

^{3}H-Thymidine, ^{125}I-IDU and visual counting assays. Either ^{3}H-thymidine (Amersham/Searle Corp.; 26 Ci/mmole) or ^{125}I-5-iodo-2′-deoxyuridine (Amersham/Searle Corp.; 0.37 mCi/0.04 mg) was added to MEM-10%NCS and the final concentration of either isotope was adjusted to 10 μCi/ml. Fluorodeoxyuridine (10^{-6} *M*) was added to the ^{125}I-IDU medium to inhibit thymidylate synthetase (14). 10,000 Sa I tumor cells were dispensed into each well of a Microtest II plate, 0.1 ml of "hot" medium was added/well, and incubated for 18–24 hr at 37°. Following this the labeled cells were washed twice with HBSS and 0.2 ml MEM-10%NCS was added/well. In the ^{3}H-thymidine assay the medium was supplemented with an excess of "cold" thymidine (9×10^{-6} *M*). The ^{125}I-IDU plates were processed as described in the section on the ^{99m}Tc microcytotoxicity assay. The ^{3}H-thymidine plates were processed as follows. After removing the admixed lymphocytes by washing, the cells were solubilized in 0.1 *M* Hyamine hyroxide, placed in vials to which was added 10 ml of scintillation cocktail containing 42 ml of Spectrafluor (Amersham/Searle Corp.)/liter of toluene. Visual enumeration of surviving cells was carried out after removing admixed lymphocytes by washing, staining the remaining adherent tumor cells with 0.1% crystal violet and then counting under an inverted microscope (18). 10,000 Sa I tumor cells labeled with ^{3}H-thymidine gave 1000–4000 cpm, while 500–2000 cpm were recorded per 10,000 ^{125}I-IDU labeled cells.

Results and Discussion. C57Bl/6 mice were inoculated subcutaneously with 20 × 10^6 Sa I cells on Day 0 and tumor growth was followed at 3 day intervals. Maximum size was attained by 8 days and complete rejection had occurred by 23 days. The development of immunity to Sa I, as determined by the ^{99m}Tc microassay, followed the growth and rejection of the tumor but with a 2 to 4 day delay. Analysis of the data in Table I indicates that cellular immunity could first be detected by Day 7, peaked at Day 10 and then progressively declined. Sera taken from tumor-bearing mice on Days 10 and 17 significantly augmented the ability of nonimmune control lymphocytes to reduce target cell survival. This phenomenon has been reported to

TABLE III. Alloimmune Reactivity of C57Bl/6 Mice to Sarcoma I 17 Days Following Tumor Challenge.

Test conditions[a]	Relative % target cell survival ± SE[b]			
	^{99m}Tc	^{3}H	^{125}I	Visual
Control lymphocytes	99.5 ± 10.7	52.8 ± 4.4	81.5 ± 3.1	82.6 ± 4.7
Test lymphocytes	67.1 ± 4.1	70.2 ± 5.7	49.3 ± 4.2	77.5 ± 3.8
Control lymphs + test sera	83.9 ± 3.0	32.2 ± 1.9	80.5 ± 5.5	63.1 ± 3.8
Test lymphs + test sera	64.5 ± 5.3	24.1 ± 2.0	61.4 ± 5.2	46.6 ± 3.0
Control lymphs + control sera	106.1 ± 4.4	22.4 ± 2.4	100.0 ± 8.8	
Test lymphs + control sera	47.1 ± 4.7	39.7 ± 2.9	66.1 ± 3.4	
Control sera + Complement	61.1 ± 2.5	71.7 ± 3.4	43.2 ± 2.6	84.3 ± 3.0
Test sera + complement	67.9 ± 4.6	61.1 ± 3.1	40.3 ± 3.0	37.7 ± 3.8

[a] Either control or test lymphocytes alone or in combination with sera were added to 10,000 ^{99m}Tc labeled target cells and then incubated for 40–48 hr at 37°. Control lymphocytes and sera were obtained from normal nontumor bearing C57Bl/6 mice.

[b] This is the ratio of the mean cpm of any control or test group compared to the mean cpm of 10,000 labeled target cells alone. Reactivity of test mice following subcutaneous inoculation of 20 × 10^6 SaI cells was determined by the technetium-99m (^{99m}Tc), tritiated thymidine (^{3}H) ^{125}I-iodo-deoxyuridine (^{125}I) isotopic assays and visual counting of surviving cells.

be independent of exogenous complement but to require viable lymphocytes (21, 22). Sera from tumor-bearing mice did not potentiate the cytocidal capacity of sensitized lymphocytes, nor were they cytotoxic in the presence of complement. This lack of complement dependent antibody-mediated cytotoxicity is consistent with earlier reports that sarcomas were generally resistant to such injury (23, 24).

Data from the ^{99m}Tc microassay on Days 10 (Table II) and 17 (Table III) have been compared with those obtained by the ^{3}H-thymidine and ^{125}I-IDU isotopic assays and with the visual counting of surviving cells. Ten days after tumor challenge all three radioisotopic assays revealed an approximate 60% difference in relative percentage target cell survival when the effects of test and control lymphocytes were compared with one another. In contrast to this, no significant differences were noted by the visual counting of surviving cells. This was attributed to the washing away of target cells in the control lymphocyte wells. Serum-mediated augmentation of the cytocidal capacity of nonimmune control lymphocytes was not detected by the ^{3}H-thymidine assay. This is attributed to the incorporation of ^{3}H-thymidine by admixed lymphocytes, thereby giving erroneously high counts for surviving cells. Alloimmune reactivity of C57Bl/6 mice 17 days after tumor challenge was detected by the ^{99m}Tc and ^{125}I-IDU radioisotopic assays with a 32% difference in relative percentage target cell survival between test and control lymphocytes. Assays based on the visual counting of surviving cells and the incorporation of ^{3}H-thymidine failed to reveal these differences. In all experiments only the ^{125}I-IDU assay approximated the sensitivity and reproducibility of the ^{99m}Tc microassay. The data obtained with the ^{3}H-thymidine isotopic assay and by visual counting of surviving cells were neither as accurate nor reproducible as that obtained with the ^{99m}Tc assay.

The principle source of error in the ^{3}H-thymidine assay appears to be due to the incorporation of released isotope by admixed lymphocytes, and either incomplete removal of these lymphocytes with gentle washing and/or the removal of labeled target cells with vigorous washing. The washing of the target cells is also critical in the visual counting assay. If this is done too vigorously surviving target cells will also be removed, and if done too gently target cells are obscured by lymphocytes and cannot be accurately counted. These problems do not occur with the ^{99m}Tc assay since there is no need to remove admixed lymphocytes and only gentle washing is required to remove detached, in-

jured target cells. The rapid labeling time and high affinity of ^{99m}Tc are significant advantages over ^{125}I-IDU. The short half-life is adequately compensated for by the high labeling efficiency and specific activity of the isotope. Finally, since ^{99m}Tc is the most widely used radioisotope in clinical scanning, it should be readily available at little or no cost in hospitals where diagnostic scanning is performed.

Summary. The ^{99m}Tc microcytotoxicity assay is a simple and sensitive means of assessing cell-mediated immunity *in vitro*. The short labeling time and high binding affinity of the nuclide are significant advantages over other radioisotopic assays which have been described. The short half-life is adequately compensated for by the high labeling efficiency and specific activity of the isotope.

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