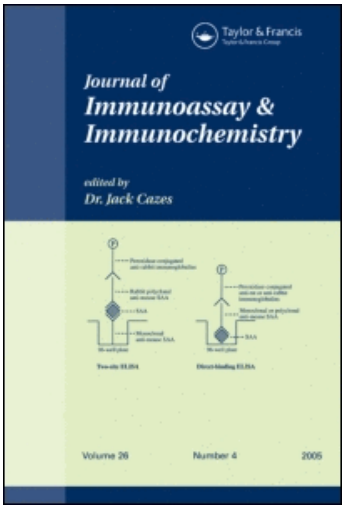

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Cell Based Radioimmunoassays to Quantitate the Immunoreactivity of TNT Monoclonal Antibodies Directed Against Intracellular Antigens

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CELL BASED RADIOIMMUNOASSAYS TO QUANTITATE THE IMMUNOREACTIVITY OF TNT MONOCLONAL ANTIBODIES DIRECTED AGAINST INTRACELLULAR ANTIGENS.

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ABSTRACT

Direct and indirect radioimmunoassay (RIA) procedures to determine the amount of binding of a mouse monoclonal antibody (MoAb) reactive with an intracellular antigen present in human cells are described. In these RIAs, mouse IgG2a MoAb, designated as Tumor Necrosis Treatment (TNT-1) antibody, paraformaldehyde/acetone fixed cells, and Sephadex beads were used to standardize the assay conditions. In the direct RIA, 83% of the ^{125}I-labeled TNT-1 MoAb was bound to the target cells within 30 min after the addition of reagents. The amount of binding of the MoAb was directly proportional to the amount of antigen present in the assay. When the direct RIA was carried out using different types of target cells, ^{125}I-labeled TNT-1 MoAb showed greater than 70% binding. In the indirect RIA, the amount of binding of secondary ^{125}I-labeled goat anti-

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mouse IgG antibody to the target cells was linear. These results suggest that the indirect RIA can be used to estimate the immunoreactivity of the unlabeled TNT-1 MoAb present in crude protein preparations. Based on the results of RIAs the following two conclusions are drawn: 1) the direct RIA can be used to estimate rapidly the amount of immunoreactive TNT-1 MoAb present in ^{125}I-labeled antibody preparations and 2) the indirect RIA which estimates the amount of immunoreactivity of unlabeled TNT-1 MoAb can be used to monitor the purification and study the characteristics of the MoAb present in crude protein preparations. These methods enable the quantitative measurement of MoAbs reactive against intracellular antigens using standard RIA procedures.

(Key Words: Intracellular antigens; Monoclonal antibodies; Radioimmunoassays; Tumor necrosis treatment.)

INTRODUCTION

The sensitivity, specificity and reproducibility of the radioimmunoassays (RIAs) are known to surpass those of many types of other immunoassays described in the literature (1). RIAs are particularly known for their accuracy in estimating microquantities of a large number of clinically related compounds. RIAs have been adopted to study antigens present in various types of samples derived from different biological species by varying the assay procedure and using different types of solid phase supports to immobilize either the antigen or the antibody (2). In general, RIAs that are designed to study secreted, purified, or cell surface antigens have simple and direct assay procedures. The detection and quantitation of antigens present inside a cell by RIA, however, requires a slightly different strategy requiring prior cell permeabilization.

We describe here RIAs which quantitate the immunoreactivity of a mouse monoclonal antibody (MoAb) that reacts with an intracellular antigen present in

mammalian cells. The mouse MoAb, which belongs to IgG2a subclass, has been shown previously to localize necrotic regions of human tumor xenografts in nude mice (3,4). We refer to this MoAb as Tumor Necrosis Treatment (TNT-1) antibody because of its potential diagnostic and therapeutic use for cancer in humans. By using fixed cell targets and TNT-1 MoAb purified from mouse ascites, we have developed procedures for a direct and an indirect RIA. The objective of these RIAs is to determine the amount of immunoreactive TNT-1 MoAb present in the ^{125}I-labeled antibody preparations as well as in mouse ascites fluid. The kinetics and results of the direct and the indirect RIAs are reported below.

MATERIALS AND METHODS

Cell cultures

Different types of human tumor and hybridoma cells used in this study were routinely grown in complete RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT), L-glutamine, penicillin (100 units/ml) and streptomycin (100 ug/ml). For growth, cells in plastic flasks (Costar, Cambridge, MA) containing complete medium were incubated at 37°C in a humidified incubator containing 5% CO_2 in air. After confluence, the adherent tumor cells were separated from the substratum by a brief incubation with trypsin: EDTA solution (Gibco). After harvesting, the dislodged cancer cells and other suspension cultures were centrifuged at 400 x g for 10 min at 4°C. The supernatants were discarded, and the cell pellets were washed with phosphate buffered saline (PBS) (3 x 30 ml). The PBS washed cells were then ready for fixation as described below. Human hybridomas were prepared as previously described (5) and were used as targets after fixing as described below.

Chemical fixation of target cells

Antigen containing target cells were treated with chemical fixatives as described below (5). The PBS washed tumor and hybridoma cells (0.4 ml packed cell

volume) were dispersed in 2% paraformaldehyde (#4018, Polysciences, Warrington, PA) (20 ml, prepared in PBS) and rocked gently for 10 min at room temperature. The cells were then centrifuged, and the supernatants were discarded. To remove any residual paraformaldehyde the cell pellets were washed with PBS (3 x 30 ml). These target cells were then dispersed in acetone, precooled to -20°C, for a 3 min incubation. The cells were centrifuged and the supernatants were discarded. After washing with PBS, the cells were used as antigen in the RIAs. For long term use, the chemically fixed cells were stored in PBS containing 1% bovine serum albumin (BSA, Sigma) and 0.02% sodium azide.

Purification of TNT-1 MoAb

The method used to produce mouse ascites containing TNT-1 MoAb is as described previously (5). Starting from the ascites fluid, the details of purification of TNT-1 will be published elsewhere. Similarly, the identification of histone as antigen for TNT-1 MoAb will be published elsewhere. The final preparation of the MoAb used in the RIAs was free from host immunoglobulins and was greater than 95% free from contaminating proteins as determined by SDS-polyacrylalmide gel electrophoresis (6).

Direct RIA

To carry out direct RIA, a calculated number of target cells and a known amount of ^{125}I-labeled TNT-1 MoAb were added to tubes containing 500 ul of PBS-BSA. The final reaction volume is 800µl. After vortexing briefly, the tubes were incubated for 30 min on a rotary shaker at room temperature. At the end of incubation, 200 ul aliquot of Sephadex G-10 gel (Sigma) suspension (a 50% W/V gel beads in PBS-BSA) was added to each tube, and the assay tube was vortexed briefly. All the tubes were then centrifuged at 1000 x g for 10 min at 4°C. The Supernatants were collected in separate tubes. To each tube containing the cell-gel pellet, 1 ml of PBS-BSA was added, and the tube was vortexed. After

centrifugation the PBS-BSA washes were pooled with the supernatants that were previously obtained from the cells. The amount of labeled TNT-1 MoAb present in each cell-gel pellet and corresponding supernatant was determined using a gamma counter (LKB-2000 Model).

Indirect RIA

The procedure used for indirect RIA is essentially as described for the direct RIA, except for the following changes: The target cells were first incubated with mouse ascites fluid containing TNT-1 MoAb for 30 min at room temperature on a rotary shaker. At the end of incubation, 200 ul of Sephadex G-10 gel suspension was added to the cells in each tube. After vortexing briefly, the tubes were centrifuged and the supernatants were discarded. Each cell-gel pellet was then washed with 1 ml of PBS-BSA. After adding ^{125}I-labeled goat anti-mouse IgG antibody to the cells, each tube was vortexed and incubated on a rotary shaker for 30 min. These cells were processed further according to the procedure described above for the direct RIA.

Other procedures

TNT-1 and goat anti-mouse IgG antibodies were labeled with ^{125}I by chloramine T method (7). The iodination reaction was initiated by adding chloramine T to a solution containing 100 ug of protein and 100 uCi of ^{125}I isotope (ICN Radiochemicals, Irvine, CA). After terminating the reaction with sodium metabisulfite, the reaction mixture was chromatographed on a Sephadex G-10 gel column. ^{125}I-labeled antibody conjugates which were eluted from the column with PBS-BSA were used in the RIAs. In all of the radiolabeled antibody preparations, the percentage of ^{125}I bound to the protein was determined by precipitating the conjugates with trichloroacetic acid (final concentration, 5%). To serve as controls

red cells were isolated from normal human blood using the standard method of Ficoll-Hypaque density gradient centrifugation. The isolated red blood cells after fixing with paraformaldehyde and acetone, were used in the RIAs as antigen negative cells. Cell viability was assessed by 0.1% trypan blue dye exclusion assay.

RESULTS

Since the antigen provided in the RIAs was in the form of intact cells, the effect of chemical fixatives on the inactivation of selective permeability barrier mediated by the cell membrane was examined using trypan blue dye exclusion assay. A combination treatment, paraformaldehyde followed by acetone, resulted in 100% non-viable cells and deeply blue stained cells. Even though microscopic examination of the fixed cells revealed the presence of intact plasma membrane, the cells leaked small amounts of proteins and other materials which had absorption at 280 nm. In the chemically fixed target cells, the antigen remained stable for more than 20 days at 4°C.

Determination of optimum incubation time

A study of the optimum time required for the maximum binding of antibody revealed that immediately after mixing the assay components, ^{125}I-labeled TNT-1 MoAb bound rapidly to the antigen present in the chemically fixed target cells (Fig. 1). Maximum amount of binding of the MoAb occurred by a period of 30 min of incubation. A further increase in the time of incubation, up to 2 hrs, brought no futher change.

Direct RIA

To determine the percentage of immunoreactivity of TNT-1 MoAb after radiolabeling, a direct RIA procedure was developed. In this RIA, when a constant amount of the antibody and a varying number of target cells were used,

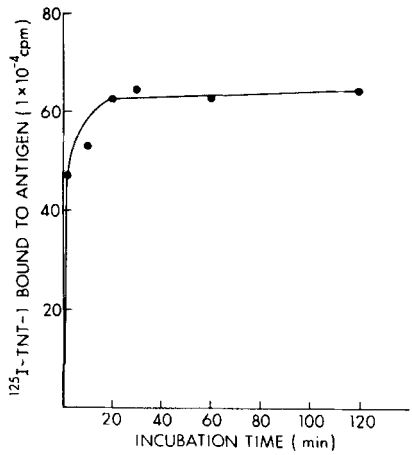

Fig. 1.

Determination of the optimum incubation time for RIA. Note the initial rapid binding of the ^{125}I-labeled TNT-1 MoAb to the antigen containing target cells. The minimum incubation time required for the maximum amount of binding of the MoAb to the target cells was 30 min.

the amount of binding of the MoAb increased linearly with increasing amount of the antigen (Fig. 2a). To supernatants obtained from the assay tubes, a constant amount of antigen containing fresh target cells were added to obtain evidence for the presence of excess of immunologically reactive TNT-1 MoAb. The amount of immunologically reactive unbound antibody was higher in the supernatants of the tubes which had few target cells and was lower in the supernatants of the tubes that had large numbers of target cells (Fig. 2b).

By employing the direct RIA, the amount of labeled TNT-1 MoAb binding to different types of cancer cells was evaluated. In general, Raji, Sultan and SK-HEP-1, which is an adeno carcinoma of liver, cells showed a higher amount of binding

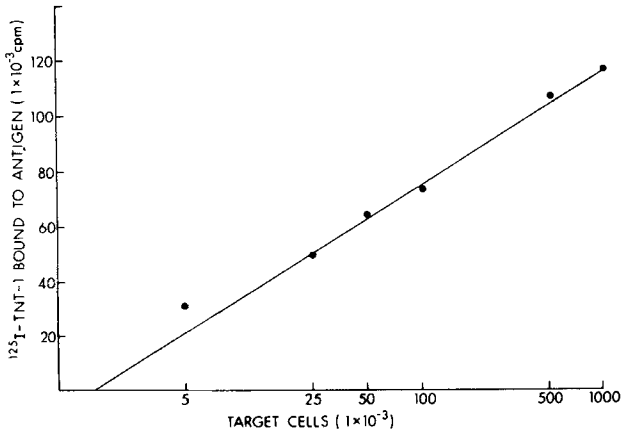

Fig. 2a.

Direct RIA. The direct RIA was carried out by using a constant amount of ^{125}I-labeled TNT-1 MoAb and varying number of antigen containing target cells. The amount of binding of the labeled MoAb increased linearly with increasing number of target cells.

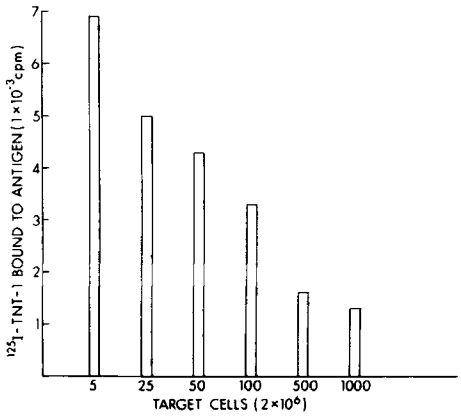

Fig. 2b.

The results demonstrate the presence of excess of immunologically reactive labeled MoAb in the supernatants obtained from the assay tubes of the direct RIA.

Table 1.

A comparison of the amount of binding of ^{125}I-labeled TNT-1 MoAb to different types of target cells.

Target cells	Binding of TNT-1 (%)
Raji	*72.5*
Sultan	*73.4*
SK-HEP-1	*70.2*
Human-human hybridoma	*82.5*
Tc-5	*75.6*
L-1210	*61.4*
TNT-1 mouse hybridoma	*59.8*
Human red blood cells	*6.1*

(Table 1). However, L-1210, which is a murine leukemia cell line, showed lower amounts of binding. Human hybridoma cells, on the other hand, which have a large nuclear diameter, showed 10% more binding of the antibody than the amount of binding of TC-5 cells, a smaller diploid parental cell line used in generating human hybridomas.

Indirect RIA

Indirect RIA, which was developed using human hybridoma cells, mouse ascites fluid, and ^{125}I-labeled goat anti-mouse IgG, was used to determine the amount of immunoreactivity of the unlabeled TNT-1 MoAb. In the indirect RIA, the target cells were incubated first with mouse ascites and then with ^{125}I-labeled goat secondary antibodies. The amount of binding of the goat antibodies increased linearly with increasing amounts of the ascites fluid that was added to the target cells (Fig. 3).

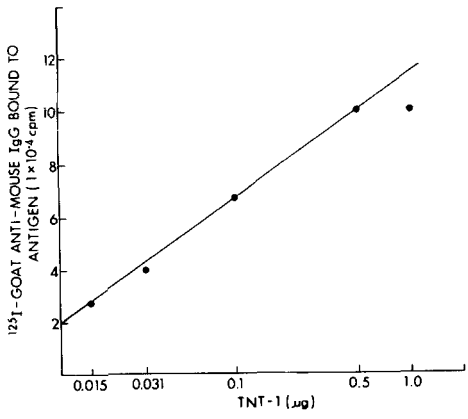

Fig. 3.

Indirect RIA. The antigen containing target cells were first incubated with mouse ascites fluid. Within the given experimental conditions, the amount of binding of ^{125}I-labeled goat anti-mouse IgG secondary antibodies was directly proportional to the amount of TNT-1 MoAb initially added to the target cells.

DISCUSSION

Detection and quantitation of antigens located inside a cell requires the fulfillment of at least the following two conditions: (a) elimination of the selective permeability barrier mediated by the plasma membrane and (b) immobilization of antigen molecules present within a cell. To meet the above described two conditions, different types of antigen containing target cells were treated with paraformaldehyde and acetone. Paraformaldehyde, as a cell fixative, exerts its action mainly on proteins by reacting with -NH2, -NH and -S-S- groups (8). Because of these effects of paraformaldehyde, several types of cellular enzymes are inactivated and many protein structures are preserved. Unlike paraformaldehyde, acetone extracts cell lipids (9) and clears the cytoplasm. When paraformaldehyde

and acetone treated cells were used in the RIAs, successful interaction of TNT-1 MoAb with the antigen present in the target cells was obtained. This result suggests that the procedure used in preparing the target cells has fulfilled both of the above described criteria. Therefore, the experimental conditions reported in this article may be applied to study viral, bacterial and other cellular antigens present in mammalian cells provided the antigen under study is not destroyed by the fixation procedure.

In several types of RIAs, centrifugation is used as an essential step in isolating the final assay product. We have also employed centrifugation as a critical step in separating the unbound ^{125}I-labeled TNT-1 MoAb from the antigen bound antibody. However, one of the technical difficulties that we have experienced in the use of chemically fixed target cells is the lack of formation of a sticky pellet at the end of centrifugation. As a result, during the aspiration of clear supernatant an indefinite number of cells are lost from the pellet because of cell floculation. To minimize this type of loss of cell bound labeled MoAb, we have added Sephadex G-10 gel beads to the reaction mixture in the direct as well as indirect RIAs. These beads which are several times larger than mammalian cells can physically trap and decrease the loss of cells during the washing procedure. Sephadex beads are hydrophylic, and therefore, can be easily equilibrated with different types of buffers that are generally used in the RIAs. Antibodies and cells which are excluded from the pores of G-10 beads can freely interact in the void volume. Furthermore, Sephadex beads have smooth outer surfaces, and therefore cause little damage to cells when the reaction mixture in the assay tubes is vortexed. Sephadex is also known to show no non-specific binding to cells or proteins. All the above described properties of Sephadex beads have helped in decreasing the tube to tube variability of counts in the RIAs.

Depending upon the assay strategy, antigen used in the RIAs is either a soluble protein, a cell surface molecule, or a biological molecule that is immobilized to a plastic surface. In general, use of chemically fixed cells as a source of antigen simplifies the methodology of RIAs. The added advantage in the use of intact cells as antigen is that one can save time and resources that are generally spent for purifying an antigen from a crude protein extract. Also, use of cells is a good alternative to the use of some types of isolated antigens in RIAs, particularly in circumstances where the nature of antigen is not known, where the target molecule is a complex protein containing several sub-units, and where the isolated antigen has low or no affinity to bind to solid phase supports. Moreover, it is easy and inexpensive to grow cells in large quantities, and after chemical fixation, it is convenient to use them as a source of antigen over a long period of time. A disadvantage in this type of cell based antigen preparation, however, is that chemical fixatives such as paraformaldehyde and acetone are known to destroy or alter the three dimensional structure of some of the macromolecules of cells. Another disadvantage in the use of whole cells is that the RIA can not quantitate separately the biologically functional antigen from physiologically inactive, precursor or breakdown products of the antigen. This particular disadvantage can be attributed to the antibody itself because the antibody can not distinguish different forms of an antigen molecule based on function.

TNT-1 MoAb has been shown to localize efficiently human tumor xenografts in athymic mice (3). This ability for tumor localization demonstrates that TNT-1 is a useful diagnostic and therapeutic agent for cancer in humans and animals. To obtain successful images in patient or animal models, it is important to quantitate the immunoreactivity of the radiolabeled antibody before in vivo administration. Beacuse of this requirement, it became necessary to develop a quantitative RIA

rather than an enzyme linked immunosorbent assay for the TNT-1 MoAb. By analyzing the radioactivity present in the blood drawn at different time intervals from patients, it is possible to determine the rate of clearance and breakdown products of the antibody in the serum. Development of an RIA which determines rapidly the percentage of immunoreactivity of the antibody also decreases the duration time that one spends before injecting the freshly labeled antibody into patients. The RIA procedure reported above is highly suitable for the clinical studies of TNT-1 MoAb in human subjects. This assay with few modifications can be adopted in many clinical and research laboratories for the study of other types of intracellular antigens.

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REFERENCES

Berson, S.E. and Yallow, R.S. Kinetics of Reaction Between Insulin and Insulin Binding Antibody. *J. Clin. Invest. 1957;36:873*

Ling, C.M. and Overby, L.R. Prevalence of Hepatitis B Virus Antigen as Revealed by Direct Radioimmune Assay with ^{125}I-Antibody. *J. Immunol. 1972; 109:841*

Epstein, A.L., Chen, F.M. and Taylor, C.R. A Novel Method for the Detection of Necrotic Lesions in Human Cancers. *Cancer Res. 1988;48:5842*

Chen, F-M., Taylor, C.R. and Epstein, A.L. Tumor Necrosis Treatment of ME-180 Human Carcinoma Cervical Carcinoma Model with 131-I labeled TNT-1 Monoclonal Antibody. *Cancer Res. 1989; 49:4578*

Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I. Two New Monoclonal Antibodies (LN01, LN-2) Reactive in B5 Formalin-fixed, Paraffin-embedded Tissues with Follicular Center and Mantle Zone Human B Lymphocytes and Derived Tumors. *J. Immunol. 1984; 133:1028*

Laemmli, U.K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage, T4. *Nature 1970; 227:680*

Hunter, W.M. and Greenwood, F.C. Preparation of ^{131}I-Labeled Human Growth Hormone of High Specific Activity. *Nature 1962; 194:495*

Sharma, A.K. and Sharma, A. Chromosome Techniques. Butterworth, Boston. 1980; P. 36

Okumuki, K. (1961) Denaturation and Inactivation of Enzyme Proteins. *Adv. Enzymol. 1961; 23:29*