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Labelling of Mouse Alloantibody with Tritiated DL-Alanine

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Summary. Radio-iodination is not a satisfactory method of labelling mouse antibody, which is peculiarly susceptible to destruction during the process of iodination. An alternative which causes very little loss of antibody is the attachment of a tritium-labelled amino acid to mouse γG by peptide linkage. This is accomplished by reaction of γG with DL-alanine *N*-carboxy anhydride under mild conditions. The method is applicable to estimation *in vitro* of the relative amounts of *H*-2 antibody absorbed by viable cells, and of the relative amounts of *H*-2 antigen on viable cells. Non-specific uptake is virtually eliminated by: (i) prior absorption of the labelled product *in vivo*, (ii) pre-incubation of the cells in 20 per cent foetal bovine serum and its inclusion in the suspending medium, and (iii) performance of absorption procedures in the cold.

INTRODUCTION

The identification of mouse histocompatibility antigens *in vitro* rests mainly on two methods: haemagglutination (see Snell and Stimpfling, 1966) and cytolysis by antibody and complement (Gorer and O'Gorman, 1956). Both techniques, while unsurpassed in specificity, are essentially qualitative, although they can be used in a quantitative manner. Thus different antisera can be compared for their content of haemagglutinating or cytotoxic antibody by titration against a standard test cell, and the relative amounts of antigen on different cell populations can be estimated by quantitative absorption of antibody. The latter, however, is exacting and laborious if a high degree of accuracy is needed (Boyse, Stockert and Old, 1968), so much so in fact that its use is sometimes precluded for reasons of impracticability. For the quantitative determination of surface antigens on viable cells, the obvious choice is radiolabelling, i.e. the introduction of a radioactive isotope in to the γ-globulin fraction of an antiserum. The most widely applied version of this principle is radio-iodination (McFarlane, 1958), which has been successfully applied to many proteins, including antibodies (Hunter and Greenwood, 1962; Hunter, 1967). However, iodination of *mouse* antibodies has met with little success in our hands and in the hands of other investigators, because specific binding activity as well as cytotoxicity is diminished drastically in the process of iodination. This is an idiosyncrasy of mouse antibody.

While low cost and convenience of handling favour the use of ^{125}I and ^{131}I, tritium is an alternative well worth considering because of its longer half-life (12·26 years) and its low emission energy—it is a β-emitter whereas ^{125}I and ^{131}I are β- and γ-emitters. For protein molecules the ideal tritium carrier would be an amino acid, attached by a peptide bond.

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Synthetic protein chemistry offers many ways of establishing peptide bonds between an amino acid and a polypeptide chain. Among these, the reactions between *N*-carboxy amino acid anhydrides (Leuch's anhydride) (Leuchs, 1906) and peptide chains, resulting in polymerization of short peptides as side chains onto the starter molecules (Katchalski, Sela, Silman and Berger, 1964), are particularly attractive, for three reasons:

(1) The reaction with protein is carried out under very mild conditions (pH 7·0 in the cold).

(2) It is possible to regulate the length of the attached peptide chain and so to control the extent of labelling.

(3) Binding of amino acids by peptide bonds is unlikely to cause major changes in the conformation of the antibody molecule. It has indeed been demonstrated that polymerization of DL-alanine to rabbit antibody does not affect its specific activity (Fuchs and Sela, 1965). These considerations are of paramount importance in the labelling of mouse antibody, which is unusually susceptible to damage under the conditions of iodination.

We report here the successful labelling of mouse alloantibody with tritiated DL-alanine *N*-carboxy anhydride, and its application for the quantitative determination of *H*-2 (histocompatibility *H*-2) antigen on viable cells *in vitro*.

MATERIALS AND METHODS

H-2 *alloantisera*

(1) Strain A (H-2^a) anti-C57BL (H-2^b) ascites leukaemia EL4. (2) C57BL/6 anti-A strain, leukaemia ASL1 (H-2^b anti-H-2^a). (3) C57BL/(H-2^k) (congenic line derived by us from 13th haemagglutination-typed backcross of AKR to C57BL/6) anti-C57BL leukaemia EL4 (H-2^k anti-H-2^b).

Preparation of γG

Method of (Kekwick, 1940). Briefly, to mouse serum at room temperature sodium sulphate was added to a final concentration of 20 per cent (200 mg Na_2SO_4/ml). The precipitate was washed twice with 20 per cent sodium sulphate solution, and dissolved in phosphate buffered saline (PBS) to about half the original serum volume. The γG was again precipitated, this time by 18 per cent sodium sulphate concentration; the precipitate washed twice with 18 per cent sodium sulphate, redissolved in PBS and dialysed against 0·05 M sodium phosphate buffer, pH 7·0, in the cold. The preparation so obtained consists of over 80 per cent γG, estimated by ring precipitation in agar. Immunoelectrophoresis against *rabbit anti-mouse serum* revealed besides a strong γG line two lines in the β-region. To preserve as much antibody as possible no further purification was attempted.

Protein determination

According to the Lowry method; with Kjeldahl standardized mouse γG (purified by DEAE chromatography) as the reference.

Labelling of mouse antibody

Generally tritiated DL-alanine *N*-carboxy anhydride (2·7 C/mM) in ethyl acetate (Humphrey, Askonas, Auzins, Schechter and Sela, 1967) was obtained from International

Chemical and Nuclear Corporation (City of Industry, California). At a concentration of 1 mc/ml, this reagent is stable for at least 6 months at −70° under anhydrous conditions. The mouse γG preparation was diluted with 0·05 M phosphate buffer, pH 7·0, to a final concentration of 5 mg/ml and the solution of DL-alanine-^{3}H *N*-carboxy anhydride added in the cold with vigorous stirring (ratio of 50 μl, equivalent to about 50 μc/ml γG solution). After 2 hours the reaction mixture was dialysed against three changes of PBS in the cold.

Preliminary absorption of ^{3}H-labelled antiserum in vivo

This step is necessary to remove labelled components that are taken up by cells non-specifically. All antisera were absorbed *in vivo* in mice of the *H*-2 type of the strain in which the antiserum was produced (e.g. A strain (*H*-2^a) mice for the antibody *H*-2^a anti-*H*-2^b, and *H*-2^k mice for *H*-2^k anti-*H*-2^b). About 1 ml of the labelled mouse γG preparation per 30 g body weight was injected intravenously. After 3–4 hours the serum was recovered by cardiac puncture, the mouse being perfused through the heart to increase recovery of labelled antibody. This absorbed serum was stored at −70°.

Specific uptake of absorbed ^{3}H-labelled mouse alloantibody by cells

Viable cells were first incubated for 1 hour in the cold in medium 199 containing 20 per cent heat inactivated foetal bovine serum (FBS), to saturate the cells with protein, and thus further reduce non-specific uptake of labelled γG. In the procedures that follow the medium was 199 (containing no phenol red*) with 5 per cent FBS. Viable cells (10×10^6) were incubated in 0·2 ml of antiserum in siliconized tubes (10× 75 mm) for 45 minutes in the cold, with frequent mixing. After centrifugation in the cold (3 minutes at 100 ***g***, 3 minutes at 200 ***g*** and 3 minutes at 500 ***g***) the cells were washed with 2 ml of medium twice, resuspended in 2 ml of medium and re-counted. In satisfactory tests, 85 per cent or more of the cells were seen to be viable (trypan blue exclusion). They were spun down at 750 ***g*** and processed for scintillation counting.

Determination of radioactivity by liquid scintillation counting

Collecting the cells on millipore filters produced inefficient scintillation counting, losses of counts being attributable to absorption of emitted β-particles by the cells themselves. The cells were therefore dissolved, thereby allowing maximum counting efficiency. The wet cell pellets were digested in 0·1 ml hyamine 10× (Packard Instrument Corp.) at 65° for 30 minutes. The residues were dried *in vacuo* over calcium chloride, dissolved in 3 drops of absolute methanol, dried once more, and then redissolved in 3 drops of methanol. This procedure was found satisfactory for removing traces of water that reduce the counting efficiency. After neutralization by 15 μl of glacial acetic acid, the contents of each tube were transferred with 10 ml of scintillation liquid [0·6 per cent Butyl-PBD (Packard Instruments) in toluene] to vials, and counted in a Packard liquid scintillation counter, model 3375.

RESULTS AND DISCUSSION

The polymerization of DL-alanine *N*-carboxy anhydride is started by free amino groups, e.g. by ε-amino groups of lysine residues of proteins (Katchalski *et al.*, 1964). Thus, poly-alanyl or oligo-alanyl chains are attached at several sites on the protein molecule serving

* Phenol red produces colour quenching in liquid scintillation counting.

as a vehicle for the tritium label. The length of the side chains, and concomitantly the degree of radiolabelling, can be increased by raising the proportion of anhydride added to the γG solution; in practice, an upper limit is set by various side reactions which may consume more than 90 per cent of the costly anhydride. These unfavourable side reactions arise mainly from hydrolysis of the anhydride, yielding free DL-[^{3}H]alanine. This in turn triggers polymerization of unreacted anhydride to form free poly-DL-[^{3}H]alanine.

The majority of unbound tritiated compounds (free DL-alanine and poly-DL-alanine) can be separated from tritiated γG by dialysis. It is possible to remove the last traces of unbound label by chromatography on DEAE-Sephadex. However, this step proved destructive to mouse alloantibody, causing considerable loss of titre, and was therefore abandoned. Instead, the dialysed ^{3}H-alanylated γG was absorbed by injection into mice intravenously and recovery by cardiac puncture. This serves three purposes: (1) free

TABLE 1
CYTOTOXIC TITRES OF THE *H*-2^k ANTI-*H*-2^b SERUM PREPARATIONS

Preparation	Titre
Untreated serum	512*
γG preparation at 2·5 mg/ml	128
[^{3}H]γG	128
[^{3}H]γG preparation absorbed *in vivo*	64

* Fifty per cent end-point against C57BL/6 lymphocytes (*H*-2^b).

DL-alanine and its polymers are rapidly eliminated from the blood, (2) partly de-graded molecules that are prone to non-specific adsorption to cell surfaces are selectively removed, and (3) the antibody is stabilized by its re-inclusion in complete serum. Mouse *H*-2 alloantibody showed no lowering of cytotoxic titre as a result of the labelling procedure (Table 1); and a satisfactorily high proportion of the antibody was recovered after absorption of the γG preparation *in vivo*, the reduction of titre by one tube being accounted for primarily by dilution during perfusion of the mouse.

SPECIFIC UPTAKE BY VIABLE CELLS

Fig. 1 shows binding of the ^{3}H-alanylated *H*-2^a anti-*H*-2^b antibody to ascites leukaemia cells of *H*-2^b type, the control leukaemia cells of *H*-2^a type remaining virtually negative. The reciprocal antiserum ^{3}H-alanylated *H*-2^b anti-*H*-2^a was positive on the *H*-2^a cells and negative on the *H*-2^b cells (Fig. 1). The uptake of antibody on positive cells was proportional to the antibody concentration in a linear fashion but in antibody excess the uptake approached saturation. ^{3}H-alanylated γG of unimmunized mice was bound by neither cell. Specificity was confirmed in another experiment in which ^{3}H-alanylated *H*-2^a anti-*H*-2^b was absorbed *in vivo* in C57BL/6 (*H*-2^b) mice; this completely abolished its binding capacity for *H*-2^b lymphocytes. In a further experiment (Fig. 2) *H*-2^b ascites leukaemia cells EL4 were incubated with excess unlabelled *H*-2^a anti-*H*-2^b and subsequently exposed to labelled antibody from the same serum. The uptake of radioactivity was blocked by about 80 per cent, also indicating the specificity of the reaction.

On the few occasions when undue non-specific absorption of labelled γG was observed, this was reduced to the usual low level by measuring uptake in the cold and by adding FBS (see 'Materials and methods'). Some of the residual non-specific uptake may be due

to pinocytosis of labelled protein, which would be reduced at lower temperature. Again, the non-specific absorption of antibody sometimes seen in conventional absorption tests with viable cells, particularly when high dilutions of antiserum are being absorbed, can be proportionally reduced by the addition of normal mouse serum (unpublished observation); cells evidently take up a certain amount of γG regardless of its specificity. Consequently, pre-incubation of test cells with FBS, and performance of the test in the cold, were adopted as standard conditions.

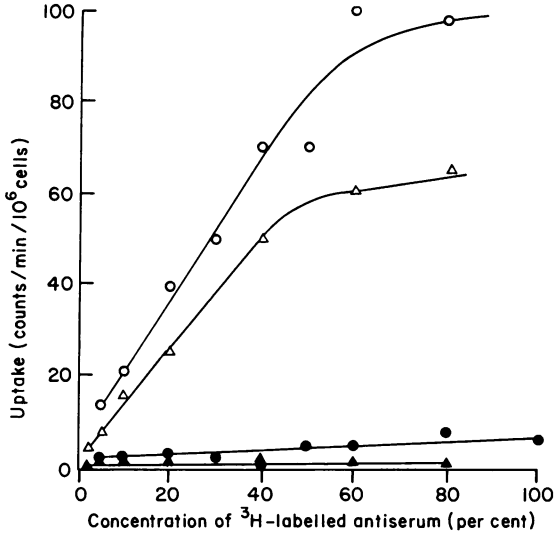

FIG. 1. Specific uptake of ^{3}H-alanylated *H*-2 antibody by leukaemia cells. Uptake of labelled *H*-2^a anti-*H*-2^b serum (circles) and labelled *H*-2^b anti-*H*-2^a serum (triangles) by C57BL ascites leukaemia EL4 cells (*H*-2^b) and strain A ascites leukaemia RADA1 cells (*H*-2^a). ○, EL4 (*H*-2^b); △, RADA1 (*H*-2^a); ●, RADA1; ▲, EL4.

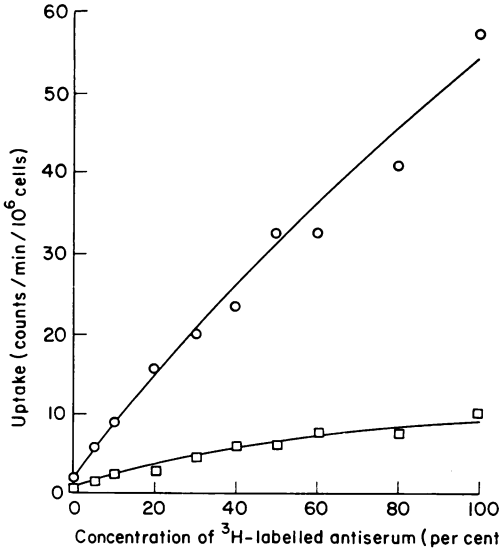

FIG. 2. Specificity: pre-incubation of (*H*-2^b) ascites leukaemia cells EL4 with unlabelled *H*-2^a anti-*H*-2^b serum blocks, their uptake of radioactivity from ^{3}H-alanylated *H*-2^a anti-*H*-2^b serum. ○, Pre-incubated with non-immune serum; □, pre-incubated with *H*-2^a anti-*H*-2^b.

KINETICS OF BINDING OF ANTIBODY TO CELL SURFACE ANTIGENS

Although an extensive study was not undertaken, some relevant information was obtained from the following experiment:

The time–temperature relationship was investigated by measuring the uptake of labelled *H*-2^a anti-*H*-2^b on *H*-2^b lymphocytes (Fig. 3). For this, constant numbers of cells were incubated with labelled antibody at 0°, 20° or 37°. At intervals, aliquots were withdrawn, the cells spun down in the cold, washed and radioactivity determined. In the experiment shown in Fig. 3 the concentration of *H*-2 antibody was less than saturating. Under these conditions, total uptake was not significantly influenced by temperature, but *rate* of uptake was considerably affected—45 minutes required for complete uptake at 0° *versus* 10 minutes at 37°. This accords with general experience in physical chemistry; the primary factor is probably the diffusion rate of antibody in the liquid phase.

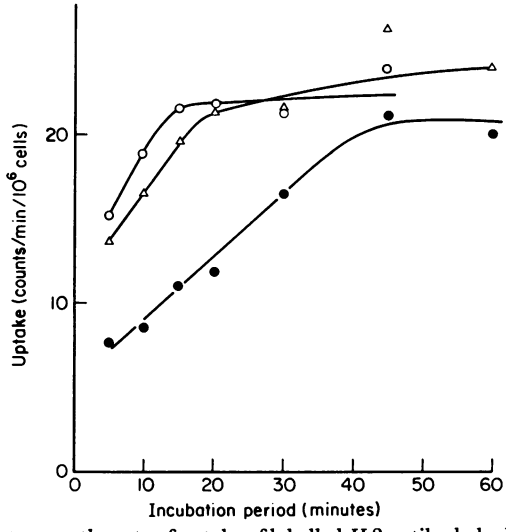

FIG. 3. Effect of temperature on the rate of uptake of labelled *H*-2 antibody by lymphocytes. Antiserum: C57BL/6 (*H*-2^k) anti-C57BL/6 (*H*-2^b); lymphocytes: C57BL/6 (*H*-2^b). ●, 0°; △, 20°; ○, 37°.

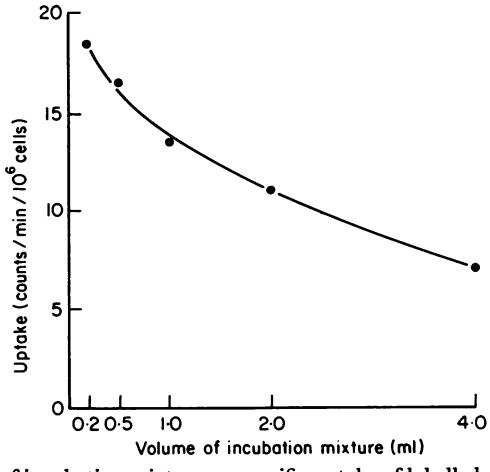

FIG. 4. Effect of volume of incubation mixture on specific uptake of labelled *H*-2 antibody by lymphocytes. Antiserum: C57BL/6 (*H*-2^k) anti-C57BL/6 (*H*-2^b); lymphocytes: C57BL/6 (*H*-2^b).

As a further example of the quantitative use of the method, Fig. 4 shows that the amount of *H*-2 antibody taken up from a constant excess in 1 hour, by a constant number of lymphocytes, is proportional to the suspension volume. This is a point of some serological interest, quite different from the antigen–antibody reaction in one-phase systems, where antigen–antibody equilibrium is far on the side of the complex. We have not attempted to determine which of many possible factors is involved—time taken to reach equilibrium according to antibody concentration, kinetic equilibrium, influence of the cell population density on metabolism, and so on. A factor we believe to be prominent is steric inhibition of antibody molecules competing at the cell surface for sites that are too densely packed to accommodate an equivalent number of antibody molecules (Boyse *et al.*, 1968).

MEASUREMENT OF RELATIVE QUANTITY OF *H*-2 ANTIGENS ON DIFFERENT CELL POPULATIONS

The relative amount of *H*-2 antigen on cell populations of different kinds can be determined with fair accuracy by comparing their absorption capacities for relevant antibodies, although this is a lengthy and difficult procedure. By this criterion lymphocytes have four times more *H*-2 antigen than thymocytes. [For D-end antigens the figure is altered if TL antigens are present on the thymocyte (Boyse *et al.*, 1968).] Fig. 5 illustrates the use of tritiated antibody for this purpose. The antibody in this case (*H*-2^k anti-*H*-2^b) being prepared in congenic mice to assure its specificity for *H*-2. In using labelled antiserum for this purpose there is an obvious risk of specific uptake of non-agglutinating and non-cytotoxic antibody belonging to unknown systems, hence the importance of antisera prepared in congenic mice.

Constant numbers of cells from lymph nodes, spleen, bone marrow or thymus, or of erythrocytes, were incubated in an arithmetic series of antibody concentrations. In each case, uptake levelled off in the higher concentration, as expected from the approach to

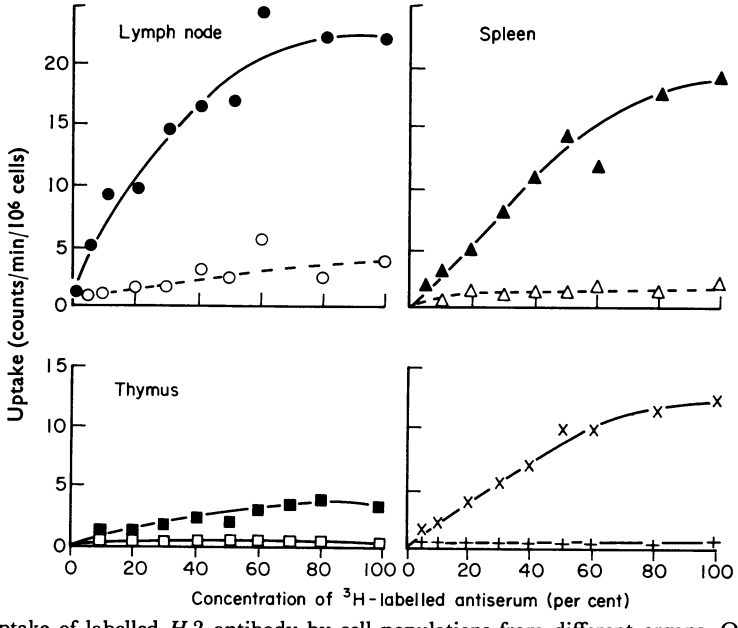

FIG. 5. Uptake of labelled *H*-2 antibody by cell populations from different organs. Open symbols, AKR (control); solid symbols, C57BL/6 (*H*-2^b). ×, C57BL/6 (*H*-2^b) bone marrow; +, C57BL/6 (*H*-2^b) erythrocytes. Antiserum: C57BL/6 (*H*-2^k) anti-C57BL/6 (*H*-2^b).

saturation, but in this and in other experiments there was a slight continuous rise with increasing excess of antibody. At least some of this may be due to a background of non-specific uptake also seen in controls (Fig. 4); non-specific uptake of presumably similar origin may affect the immunofluorescence test with viable cells described by Möller (1961). However, from other experiments now going on in this laboratory we conclude that the quantity of antibody taken up by cells depends in part on the concentration of excess antibody present (Chang, Stockert, Boyse and Old, 1969). This is in line with the conclusion that the density of *H*-2 sites on the cell surface is too great for them all to be engaged by antibody, so that there is crowding of *H*-2 antibody molecules in proportion to their concentration (Boyse *et al.*, 1968).

Fig. 5 is in line with other serological data. Thus if *H*-2 representation on lymph node cells = 100 per cent, then the figure for spleen cells is about 90 per cent, for bone marrow cells 60 per cent and for thymocytes 20 per cent. The amount of *H*-2 on red blood cells was below the level of the sensitivity of the weakly labelled antiserum used in these experiments, ranking less than 5 per cent compared with lymphocytes.

The purpose of the experiments reported here was to indicate the feasibility of labelling mouse γG in the manner described. For future routine application a number of shortcomings ought to be eliminated. Non-specific absorption at first seemed a problem, but it was later found that labelled protein becomes absorbed on the glass surface and that when cells are transferred for hyamine digestion into clean tubes, the controls are essentially negative throughout the entire range of antibody concentrations tested. A present point of concern is undue fluctuation of readings, the most likely source of this being inaccurate cell counts. An unambiguous determination of cell number is required, and labelling of the cells with a second radioisotope for this purpose is under investigation.

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