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Spontaneous Release of Cytotoxic Alloantibody from Viable Cells Sensitized in Excess Antibody

S. CHANG,* ELIZABETH STOCKERT, E. A. BOYSE,
U. HÄMMERLING AND L. J. OLD

Sloan-Kettering Institute for Cancer Research,
New York, New York 10021, *U.S.A.*

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Summary. When viable cells sensitized in excess cytotoxic alloantibody are washed and resuspended in antibody-free medium, they spontaneously release appreciable quantities of antibody. The amount released is directly proportional to the concentration of alloantibody during sensitization. Spontaneous release was observed from all cell types tested (thymocytes, lymphocytes, leukaemia cells and ascites sarcoma cells) and with all alloantibodies tested (H-2, θ and Ly-B). In preliminary tests with radio-labelled H-2 antibody, the quantity of antibody released in a period of 2½ hours was 29 per cent of the antibody originally absorbed. Dissociation at 37° was greater (or more rapid) than at 1°. When washed sensitized cells were suspended in antibody directed to an antigen closely adjacent on the cell surface to the site of attachment of the first antibody, release of the first antibody was impeded.

INTRODUCTION

During recent serological work we found it necessary to ascertain the extent to which alloantibody specifically bound to viable nucleated cells may spontaneously dissociate *in vitro*. The following two examples, from our own experience, illustrate circumstances in which this must be taken into account.

First, we have devised a method of plotting the position of cell surface antigens relative to one another which involves measuring the degree to which antibody of a given specificity, previously adsorbed to the cell surface, blocks the capacity of the cells to absorb antibody of a second specificity (Boyse, Old and Stockert, 1968). If two antigens are sufficiently close, the space available for attachment of the second antibody is restricted by the previously attached antibody, and this interference can be measured. However, dissociation of the first ('blocking') antibody from the washed sensitized cells, while these are being tested for their absorption capacity relative to the second antibody, could lead to error if the test cells of the absorption system carried both antigens. For this reason we had to ensure in every test system that antigen of the first specificity was lacking from the target cells used in the second (absorption) system.

The second example concerns *antigenic modulation* (Old, Stockert, Boyse and Kim, 1968). This is an adaptive phenotypic suppression of TL antigens which takes place when TL^+

* Present address: Dr. S. Chang, Division of Virology, National Cancer Research Institute, Tsukiji 5-Chome, Chuo-ku, Tokyo, Japan.

cells are exposed to TL antibody *in vitro* or *in vivo*. One of the problems posed by antigenic modulation is the fate of the modulating TL antibody when TL antigen disappears from the cell surface. Is the initially bound TL antibody retained by the cell or is it released? It transpires that some TL antibody *is* released into the suspending medium during modulation. But the question arises whether release of alloantibody occurs in systems other than TL and may have nothing to do with antigenic modulation *per se*.

To resolve these uncertainties, we undertook to find out whether and under what circumstances spontaneous dissociation of alloantibody is a significant factor in serological tests with viable nucleated cells.

MATERIALS AND METHODS

Abbreviations. BALB = BALB/c; C57BL = C57BL/6; 199 = medium 199.

Cytotoxic test (Boyse, Old and Chouroulinkov, 1964; Gorer and O'Gorman, 1956). Serial dilutions (0·05 ml) of antiserum were incubated with cells (0·05 ml of a suspension containing 5×10^6 cells/ml) and pooled guinea-pig serum (GPS) diluted 1/3 (0·05 ml) to provide C′. After incubation for 45 minutes at 37° the cells were counted in trypan blue to determine the dead (stained) cell count per cent. The guinea-pig serum pool was made up from animals selected for low natural cytotoxicity for mouse cells. The titres shown in the figures are 50 per cent end-points (= the dilution at which 50 per cent of the test cells were killed); in satisfactory tests with cells of all types, dead cell counts in controls incubated in either antiserum alone or complement alone never exceeded 5 per cent. The 50 per cent end-points were ascertained by plotting the dead cell count on a semilogarithmic graph and reading off the dilution indicated by a dead cell count of 50 per cent.

Antisera. These are identified in the legends to the figures, with the exception of Fig. 9. The antisera for that experiment were: (a) *Anti-H-2^a K region; (BALB/c × C57BL/6)F_1 anti C3H ascites sarcoma BP8.* (Relevant specificities include H-2.1,11,25,32). (b) *Anti H-2^b K region; C57/H-2^k* (congenic with C57BL/6) *anti C57BL leukaemia EL4 (H-2^b)*, absorbed in H-2H mice to remove H-2^b D region antibody. (Relevant specificities include H-2.22, 33). (c) *Anti H-2^b D region antibody*; the same antiserum absorbed in H-2I mice to remove H-2^b K region antibodies. (Relevant specificities include H-2.2).

The mice used for absorption, H-2H and H-2I, are crossover stocks derived from crosses between H-2^a and H-2^b mice (see Snell and Stimpfling, 1966) and so have special value in separating D-end antibodies from K-end antibodies.

Alloantigenic systems. For a description of the H-2 system of alloantigens see Snell and Stimpfling (1966), for θ see Reif and Allen (1964), and for Ly-B see Boyse, Miyazawa, Aoki and Old (1968).

Radio-labelling of H-2 antibody. For experiments of the kind illustrated in Fig. 10, H-2 antibody was labelled with tritiated alanine according to the method of Hämmerling, Shigeno, Old and Boyse (1969).

Determination of radioactivity. Supernates, or washed cells, were dissolved in Soluen-100, transferred to a toluene-based scintillation cocktail, and counted in a Packard Model 3375 Scintillation Counter. Counts per minute (cpm) were corrected for quenching effect by the automatic external standardization.

RESULTS

Influence of temperature

Fig. 1 shows an experiment in which strain A ascites leukaemia cells were incubated in

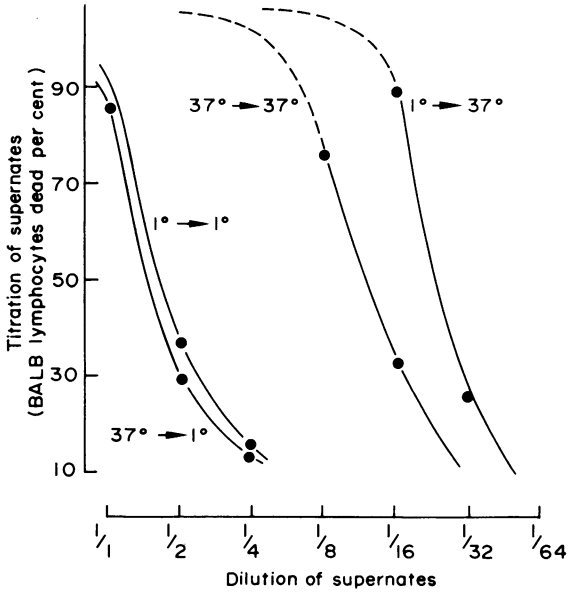

FIG. 1. Spontaneous release of H-2 antibody from washed sensitized cells: relation to temperature. Sensitization of RADAI (A strain) ascites leukaemia cells (5×10^7/ml) in excess *H-2*^{d} *anti-H-2*^{d} antiserum (12·5 per cent in 199) for 15 minutes either at 1° or at 37°. Cells washed ×3 in the cold; last wash fluid shown to be free of antibody. Sensitized washed cells resuspended (5×10^7/ml) in 20 per cent foetal bovine serum (FBS) in 199 for 1 hour either at 1° or at 37°; viability count (trypan blue) 90 per cent throughout. The four supernatant solutions were then titrated *vs* H-2^d (BALB) lymphocytes; relevant specificities H-2·3,4,8,10,13,31. Maximal release occurs at 37°, after sensitization at 1°.

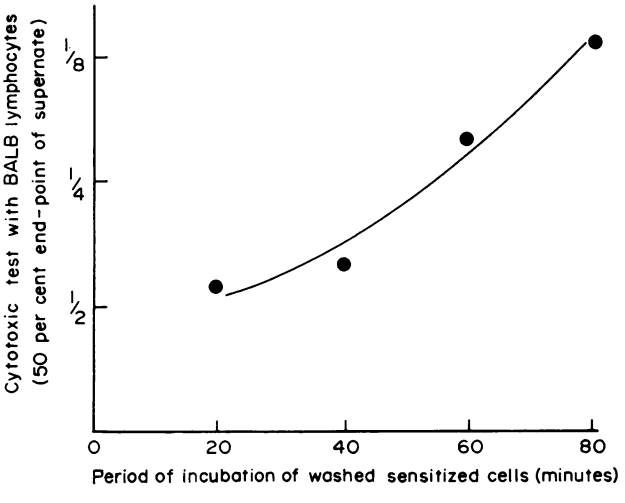

FIG. 2. Spontaneous release of H-2 antibody from washed sensitized cells: increase with time. Sensitization of RADAI ascites leukaemia cells for 15 minutes at 37°, followed by incubation of the washed sensitized cells at 37°. At the times indicated, samples of the cell suspension were removed, centrifuged, and supernates titrated for released antibody. Other details as in Fig. 1.

excess H-2 antibody either at 37° or 1°. After washing, each suspension was divided into two parts, one of which was further incubated at 37°, the other at 1°. Spontaneous release was greater at the higher temperature, and greatest from the cells which had been sensitized in the cold.

We conclude that spontaneous release is enhanced as the temperature rises to physiological levels, and that more H-2 antibody can be absorbed at lower temperatures.

Increased release of alloantibody with time

In tests of the kind illustrated in Fig. 2 the washed sensitized cells were resuspended, and the medium was titrated for released antibody at increasing intervals of time. These experiments indicate continued release of H-2 antibody over a period of 1 hour or more, with consequent increase in the concentration of released antibody in the suspending medium.

Fig. 3 shows spontaneous release of θ antibody from washed sensitized strain A ascites leukaemia (θ-C3H), most of the release occurring in the first 30 minutes or so.

Fig. 4 shows spontaneous release of antibody belonging to a third antigenic system, Ly-B, also increasing with time. This cytotoxic system is weaker than H-2 or θ, and so the results are expressed not in terms of the titre of antibody in the suspending medium but as the percentage of a standard cell suspension lysed by the undiluted suspending medium.

Influence of antibody concentration

Results such as those in Fig. 1, in which it appears that more H-2 antibody can be absorbed at 1° than at 37°, suggest that 'saturation' is a relative term. If temperature affects the degree of saturation other factors may be expected to do so also, and the most obvious of these is the concentration of excess antibody to which the cells are exposed.

Fig. 5 illustrates that the concentration of saturating antibody does indeed have a marked influence on subsequent release, presumably by influencing the degree of saturation. In this

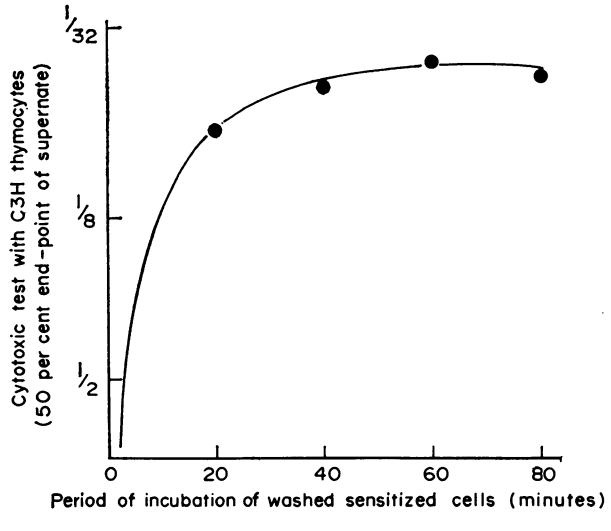

FIG. 3. Spontaneous release of θ antibody from washed sensitized cells. Cells; RADAI ascites leukaemia. θ antibody; *AKR anti-C3H thymocytes*. Test cells; C3H thymocytes. See Fig. 2 for other details.

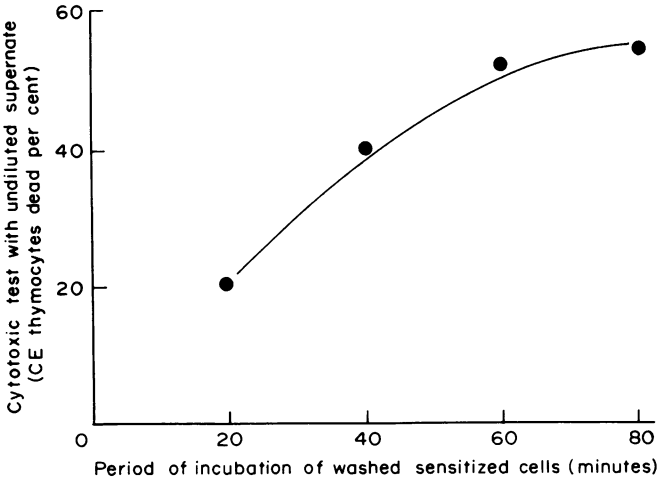

FIG. 4. Spontaneous release of Ly-B.1 antibody from washed sensitized cells. Ly-B.1 antiserum; C57*BL*/*H*-2^k (*congenic with C57BL/6*) *anti CE thymocytes*. Sensitized cells, AKR ascites leukaemia K36 (H-2^k: Ly-B.1). See Fig. 2 for other details.

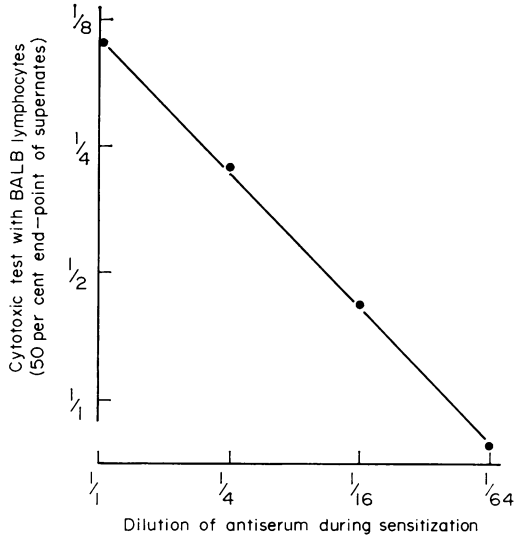

FIG. 5. Release of H-2 antibody from washed sensitized cells is proportional to the concentration of excess H-2 antibody present during sensitization. BALB ascites sarcoma Meth A cells (H-2^d; 1×10^7/ml) sensitized with four different concentrations of H-2^b *anti*-H-2^d.

	1	2	3	4
Dilution of sensitizing antibody	1/1	1/4	1/16	1/64
Residual titre after sensitization	1/024	1/64	1/16	1/2

See Fig. 2 for other details.

test a suspension of ascites sarcoma cells was divided into four parts, each being exposed to a different concentration of H-2 antibody. All four concentrations were 'saturating' in the sense that residual antibody was demonstrable in the supernatant solution after incubation. However, the titre of residual supernatant antibody was 1:1024 for the first suspension and only 1:2 for the fourth suspension. Fig. 5 shows that the amount of antibody subsequently released was directly proportional to the concentration of the saturating antibody.

We conclude that in antibody excess, an increase in the concentration of antibody in the suspending medium increases the degree of saturation of the cells which in turn increases the amount of antibody subsequently released from the cell after their removal from antibody excess.

Physical state of released H-2 antibody

The following experiment was performed to see whether there might be any marked change in the character of the antibody molecule brought about by its specific absorption to cells and its spontaneous release. The supernatant solution from 80 minutes incubation of a suspension of washed cells sensitized with *anti-H-2* was fractionated on Sephadex G-200 in the presence of added (control) H-2 antiserum of a different specificity (Fig. 6).

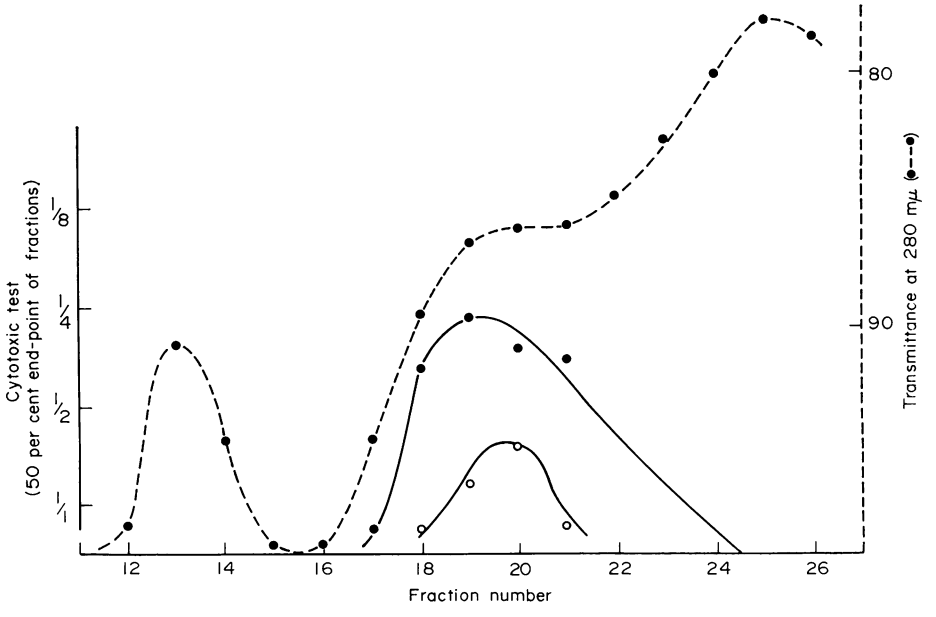

FIG. 6. Sephadex G-200 chromatography of H-2 antibody released from washed sensitized cells. RADAI ascites leukaemia cells sensitized with H-2^b *anti*-H-2^d antibody (see Fig. 2), washed ×3 in the cold, (last wash fluid was free of antibody), resuspended ($5 \times 10/ml^7$) in 5 per cent H-2^a *anti*-H-2^b antiserum (control antibody) for 80 minutes, at 37°, and spun down. 5 ml of this supernatant solution was passed through a Sephadex G-200 column 2·2cm × 40 cm, eluted with 0·1 M Tris-HCl buffer pH 8·0 containing 0·2 M NaCl. Fraction size was 2 ml. Tramsmittance monitored at 280 μ. Fractions were dialysed against isotonic phosphate buffered saline pH 7·2 for 4 hours and tested for released antibody. (●—●, cytotoxic test with BALB lymphocytes) and for control antibody (○—○, cytotoxic test with C57BL lymphocytes). The released H-2 antibody appears in the same fractions as the added (control) H-2 antibody.

H-2^a leukaemia cells were sensitized with *H*-2^b *anti-H*-2^d serum, washed and resuspended in a 1/20 concentration of the control serum *H*-2^a *anti- H*-2^b for 80 minutes. The cells were now spun out, and the supernatant solution passed through a Sephadex G-200 column 2·2 cm × 40 cm. 5 ml of the supernate was applied and eluted with 0·1 M Tris HCl buffer pH 8·0 containing 0·2 M NaCl. Eluate was collected in 2 ml fractions, dialysed against isotonic phosphate buffered saline pH 7·2 for 4 hours. The fractions were then titrated against the relevant test cells for the presence of the control antibody and of the released antibody. Fig. 6 shows approximately coincident peaks in the γG region, indicating that according to Sephadex filtration there is no physical change in the antibody consequent on its being bound to a surface antigen and subsequently released.

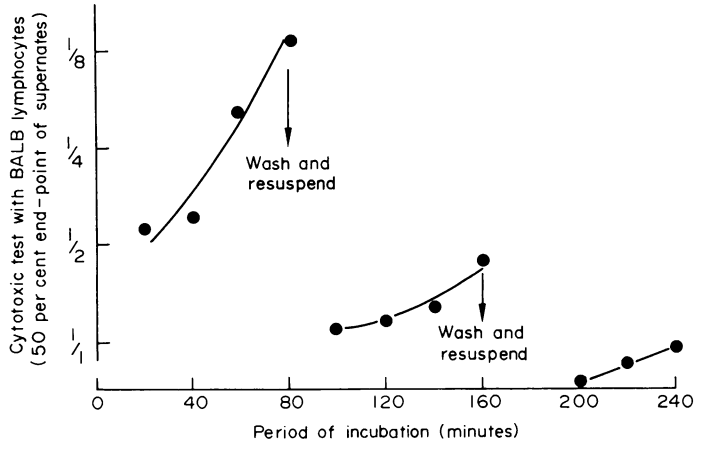

FIG. 7. Effect of additional washing and resuspension on the spontaneous release of H-2 antibody from washed sensitized cells. Experimental conditions (excepting washing and resuspension) as in Fig. 2.

Effect of repeated washing and resuspension

In Figs 3 and 4 the rate of release is seen to diminish with time. This is to be expected because the release comprises mainly the extra antibody taken up in proportion to the antibody excess (see Fig. 5). Fig. 7 illustrates that there is, as expected, considerable diminution in the amount of H-2 antibody released during consecutive periods of time after repeated cycles of washing and resuspension.

Interference between antibodies directed to neighbouring antigens

If two cell surface antigens are situated sufficiently close together, the respective antibodies compete for the limited space available for their attachment (Boyse, Old and Stockert, 1968). In order to see what effects this competition might have upon the spontaneous release of antibody, a number of experiments were carried out in which cells were sensitized with antibody of one specificity (anti A) and then resuspended either (a) in antibody of a second specificity (anti B) selected because antigens A and B were known to be close enough for antibody-competition (Boyse, Old and Stockert, 1968), or (b) in the same concentration of normal mouse serum, as control. In Fig. 8 the two specificities concerned are θAKR and θ-C3H, and the cells are heterozygous AKR/C3H thymocytes.

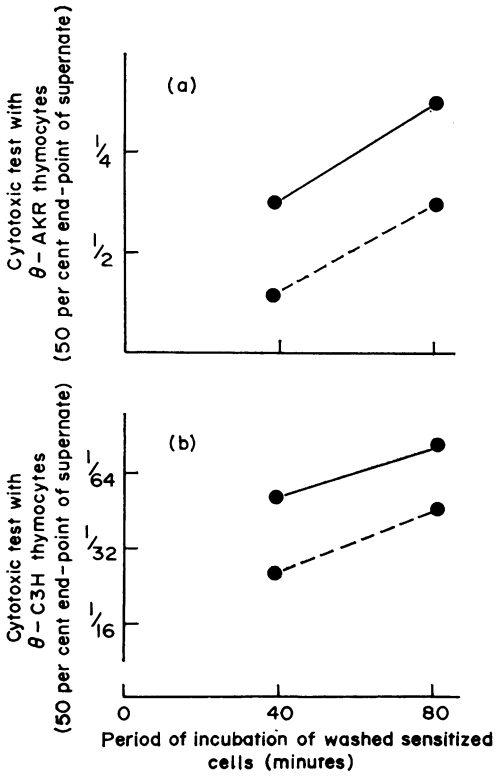

FIG. 8. Release of θ antibody is impeded by addition of antibody directed to a neighbouring antigen. For experimental conditions see Fig. 2. (A) (C57BL × AKR)F_1 thymocytes (θ-C3H/θ-AKR) sensitized with *anti-θ-AKR* serum, washed, and incubated in (1) ●- - ●, 10 per cent anti-θ-C3H serum, or (2) ●—●, normal mouse serum. Supernatant solutions tested on AKR thymocytes for released anti-θ-AKR antibody. (B) The reciprocal experiment.

Actually Fig. 8 shows two experiments, the roles of the two antibodies, anti-θ-AKR, and anti-θ-C3H being reversed in the second experiment. In both experiments the addition of the second antibody impeded the release of the first. The effect on *rate* of release, whether the *total* release would be the same over longer intervals of time, was not determined.

Fig. 9 shows a similar experiment involving H-2, with a control showing that antibody attached to a more distant site does not impede release. Heterozygous H-2^{ab} cells were sensitized with H-2 antibody directed to antigens of the K region of the H-2^a haplotype. The washed sensitized cells were suspended in (a) antibody to antigens of the K region of H-2^b; it was expected that this antibody might compete with the sensitizing antibody, (b) antibody directed to the D region of the H-2^b; it was not expected that this antibody would compete with the sensitizing antibody because D region antigens do not lie within blocking range of K region antigens (Boyse, Old and Stockert, 1968), and (c) normal serum. Fig. 9 shows that impedance of release was confined to antibody attached to the closely adjacent sites.

Transfer of antibody from sensitized to unsensitized cells

In a number of tests unsensitized lymphocytes were added to suspensions of morphc-

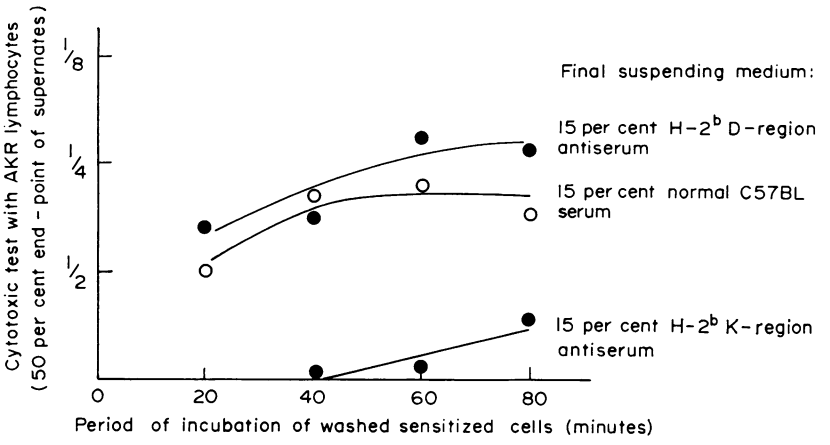

FIG. 9. Specificity of impedance of antibody release by antibody absorbed to neighbouring site. This test was based on the knowledge that in tests with comparable antisera H-2^a (K end) and H-2^b (K end) antigens are sufficiently close to produce interference between the respective antibodies, whereas the former are too distant from H-2^b (D end) sites to produce such interference (Boyse, Old and Stockert, 1968). H-2^{ba} (C57BL/6 × A)F^7 leukaemia cells (4×10^8/ml) sensitized with anti-H-2^a K-region antibody, washed, and suspended in: (a) ani-H-2^b K-region antibody, (b) anti-H-2^b D-region antibody (negative control), or (c) normal C57BL/6 mouse serum (control). (The antisera are identified under Materials and Methods). Release of antibody is impeded by antibody later attached to a closely adjacent site, but not by antibody attached to a more distant site.

logically distinct cells, such as ascites sarcoma cells, which were of the same H-2 type and had been sensitized with H-2 antiserum, washed, and resuspended. Addition of complement later caused specific cytolysis of a proportion of the added lymphocytes, indicating that they had absorbed H-2 antibody released by the sensitized cells. (For similar observations involving red cells, see Bownma, Mayer and Rapp, 1951).

Use of radio-labelled H-2 antiserum to measure the proportion of antibody released

Satisfactorily labelled H-2 antiserum became available to us only at the conclusion of this study, and only a few tests have so far been done, all of them with one antiserum *C57BL/6 (H-2^b) anti-A-strain leukaemia ASL1 (H-2^a)*, pre-absorbed with TL$^+$H-2^b cells to remove TL antibodies. In the experiment illustrated in Fig. 10, approximately 29 per cent of the antibody was released from RADA1 (H-2^a) leukaemia cells, during a period of 2½ hours after washing and resuspension.

Confirmation that the released antibody was initially bound to tell surface antigen and was not adsorbed non-specifically.

We considered the possibility that at the high concentrations of antibody used for sensitization a small proportion was adsorbed non-specifically, and that it is this fraction which is subsequently released rather than antibody specifically bound to cell surface antigen. This was investigated for H-2 by using the sera *H-2^d anti-H-2^k* and *H-2^k anti-H-2^d* and testing both antisera for release from cells of either H-2^k or H-2^d phenotype. *H-2^d anti-H-2^k* antibody was released from the H-2^k cells but not from H-2^d cells; similarly *H-2^k anti-H-2^d* antibody was released from H-2^d cells but not from H-2^k cells. Thus it seems that non-specific binding of antibody γG, if it occurs, does not measurably contribute to the antibody released.

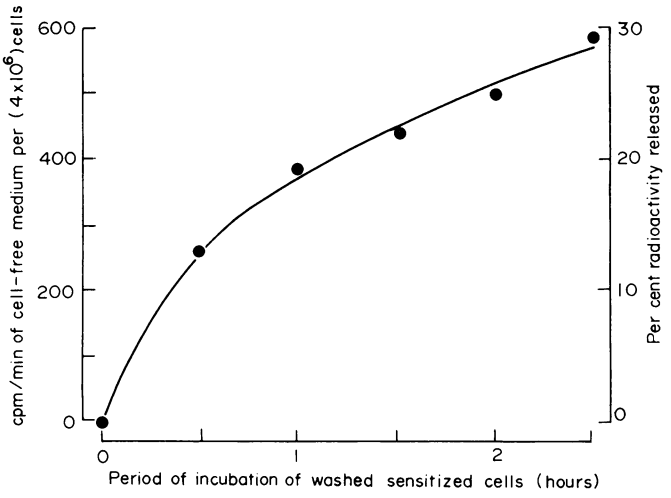

FIG. 10. Release of radio-labelled H-2 antibody from A strain leukaemia RADAI. Method: 3×10^7 cells in 2 ml Medium 199 with 20 per cent foetal calf serum added to 2 ml radio-labelled H-2^b *anti-H-*2^a serum (see Methods); incubated at 0° for 40 minutes, washed 5×, resuspended to 4×10^6/ml in six aliquots, and incubated at 37°. Cells and supernatant solution of one aliquot separated and counted every 30 minutes. The sum of counts of cells and supernatant solutions separately at each time interval was in each case adequate to account for at least 95 per cent of the initial uptake, i.e. of the initial count of washed sensitized cells; 'per cent radioactivity released' (right-hand ordinate) was calculated by dividing the count of the cell-free medium at each time interval by this initial count. Controls: As the uptake of radioactivity by H-2^b (negative control) cells was negligible, it is concluded that the only significant uptake and release of radioactivity was that associated with antibody. The radioactive supernatant medium from this experiment was incubated with (a) H-2^a leukaemia cells, or (b) H-2^b leukaemia cells; there was significant uptake of radioactivity by the former, but not by the latter.

DISCUSSION

Our purpose was to find out whether and under what circumstances spontaneous dissociation of alloantibody from sensitized viable cells might be appreciable *in vitro*. In broad terms it appears that cells that have been sensitized in excess alloantibody release readily detectible quantities when resuspended in antibody-free medium at physiological temperatures. The indications from a few experiments with radio-labelled H-2 antibody suggest that this release may approach one-third of the antibody originally adsorbed, within a period of 2½ hours. The same dissociation is presumably taking place from cells suspended in antiserum, although balanced at equilibrium by antibody uptake. Spontaneous dissociation seems relatively unaffected by the type of cell and by the specificity of the alloantibody, as far as we have tested this.

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