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The use of the enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of specific antibody from cell cultures

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Summary. The solid phase enzyme linked immunosorbent assay (ELISA) has been used to quantify anti-keyhole limpet haemocyanin (anti-KLH) antibody in the serum of KLH-immune C57Bl/6 mice. When spleen cells from immune mice were cultured overnight in ELISA microtitre wells to which KLH had been adsorbed it was found that easily quantifiable amounts of anti-KLH antibody were synthesized and were detectable. It was found further that spleen cells from KLH-primed mice, when cultured *in vitro* in the presence of KLH, transferred to KLH-labelled ELISA plates, and cultured overnight, also produced detectable levels of antibody. Levels of antibody were detectable only after 4 and 5 days of *in vitro* stimulation. A comparison was made between detectable numbers of plaque forming cells to sheep red blood cells (SRBC) in SRBC primed CBA mice and levels of antibody detected by the ELISA procedure. It was found that the sensitivities of the two tests were comparable. The applications of this technique to the study of *in vitro* antibody synthesis using soluble antigens are discussed.

INTRODUCTION

The enzyme linked immunosorbent assay (ELISA) which has been used frequently during the past decade for the serological detection of antibodies to a variety of viral or other parasitic antigens, is recognized as a test with sensitivities in the range of many radioimmunoassays (Engvall & Carlsson, 1976). The most recent advance in the use of this procedure has involved its application to microtitre wells which are used as a solid phase for antigen adsorption following which a sandwich technique is used to detect specific antibody. The developing antiserum is most frequently labelled with the enzyme alkaline phosphatase, for which there is a simple quantitative colorimetric assay (Voller, Bidwell & Bartlett, 1976). In the work reported here, we have shown the quantification of anti-KLH antibody in the serum of KLH immune C57Bl/6 mice by using this technique. The ELISA, when used in comparison with a radioimmunoassay, was found to be more sensitive in detecting specific antibody down to the 2 ng level.

While the ELISA technique has enjoyed an increasing popularity as a serological assay because of its simplicity and sensitivity, it has not, so far been applied to antibody detection in cellular immunology. To date, the main technique for detecting antibody produced by cell populations has been the plaque assay (Cunningham & Szenberg, 1968). While this procedure is sensitive and has been invaluable in studies on antibody synthesis *in vitro*, it has limitations, the most notable of which is the difficulty incurred in applying its use to a number of soluble antigens. ELISA could be useful in studies involving such antigens, for this technique depends upon the adher-

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ence of the antigen to a solid phase plastic surface on which subsequent reactions take place. The present study was undertaken to determine whether the ELISA procedure could be used to detect either *in vivo* or *in vitro* antibody production by cell populations and provide an alternative to the plaque assay for studies with soluble antigens. The results indicate that this indeed may be the case.

MATERIALS AND METHODS

Experimental animals

C57Bl/6 and CBA/J (Jackson Laboratory, Bar Harbor, Maine) of either sex and between 2 and 4 months of age were used in all experiments.

Immunization protocol

C57Bl/6 mice were primed with keyhole limpet haemocyanin (KLH) (Calbiochem) by a single subcutaneous injection of 50 μg of KLH in 50% Freund's complete adjuvant (CFA) and 0·15 M NaCl in a total volume of 0·2 ml. Primed animals were left 4–6 weeks before use. Non-immune control animals were immunized with an equivalent volume of CFA. Animals receiving a secondary *in vivo* antigenic stimulus were injected as above and killed by cervical dislocation 7 days later. Sera were collected from these animals and the spleens were removed for cell culture. CBA/J mice were immunized with a single intraperitoneal injection of 5×10^8 SRBC in 0·1 ml PBS. They were killed 5 days later and their spleens used for immunological testing.

Developing antiserum

High titre rabbit anti-mouse Ig (RAMIg) was prepared by injecting a rabbit i.m. at 2 week intervals with 50 μg of MOPC-46 P-4 derived κ chains (Levy, McMaster, Kelly, Whitney & Kilburn, 1977) in 50% CFA. The animal was exsanguinated 2 weeks after the last injection, and the serum heat-inactivated at 56° for 30 min and stored frozen. The immunoglobulin (Ig) from an aliquot of the serum was purified on DEAE cellulose according to standard methods (Campbell, Garvey, Cremer & Sussdorf, 1970). The purified Ig was adjusted to 1·0 mg per ml and linked by glutaraldehyde to alkaline phosphatase (Sigma) according to the method of Engvall & Perlmann (1971). The ratio of Ig:alkaline phosphatase was 1:5 and the final concentration of glutaraldehyde was 0·2%. This labelled antiserum was titrated against known standards and was used routinely at a dilution of 1:1000.

Preparation of soluble SRBC Antigen

Five millilitres of packed SRBC were lysed hypotonically by suspension in 10 ml of distilled water. The lysates were centrifuged at 20,000 *g* for 15 min. The pellet was washed three times in PBS and finally resuspended in 4·0 ml of PBS. This material was sonicated in a Biosonic 80 with a sonic probe at 0° for 30 s. This material was centrifuged at 30,000 *g* for 60 min, and the soluble supernatant was used for attachment to ELISA plates at 10 μg per ml.

ELISA Test

The basic test used throughout this work was carried out as described previously (Voller *et al*, 1976; Engvall & Perlmann, 1971). Briefly, 0·2 ml of KLH at 200 ng per ml in pH 9·6 carbonate buffer was attached to substrate microtitre plates (Cooke Engineering Co., Alexandria, Va., No. 1–220–29S) for 18 h at 4°. After washing with PBS-Tween buffer, sera to be tested for anti-KLH activity were added to the wells as 0·2 ml aliquots. Following incubation for 2 h at room temperature and subsequent washing, the developing enzyme-linked RAMIg at 1:1000 was added in 0·2 ml aliquots. After a further 2 h incubation and final washing with buffer, the enzyme substrate solution (Sigma 104–105) was added to each well (0·2 ml) and the enzyme substrate reaction was allowed to continue for 30 min (unless otherwwise stated) at room temperature. The reaction was terminated by the addition of 0·05 ml of 3 M NaOH to each well. The contents of each well were transferred to tubes containing 0·75 ml of distilled water and read for absorbance at 400 nm in a Beckman DBG spectrophotometer. Tests were run in quadruplicate. All sera were tested in this way; when the basic method was adapted to the culturing of antibody producing cells, it was modified in several ways (see below).

Radioimmunoassay

Anti-KLH serum and normal serum were chromatographed on DEAE to purify the IgG fraction according to the method of Dissanayake & Hay (1975). Iodination with ^{125}I was carried out according to the chloramine T procedure of Greenwood, Hunter & Glover (1963). The resulting specific activity of the anti-KLH IgG was $2{\cdot}2 \times 10^7$ c.p.m. per mg and $2{\cdot}4 \times 10^7$ c.p.m. per mg for the normal mouse IgG. After exhaustive dialysis, ELISA plates were set up

using quadruplicate samples of doubling dilutions from 1:5–1:120 of the ^{125}I-labelled materials in both the absence and presence of KLH adherent to the plates. The tests were developed as described above and absorptions at 400 nm recorded. The empty wells were subsequently cut out and counted individually in a gamma counter (Beckman Biogamma). Net counts for specific anti-KLH activity were determined by subtracting counts obtained in wells without antigen from those obtained with antigen at the equivalent dilution of Ig. The specificity of these net counts was established by our observation that labelled normal non-immune IgG gave essentially identical counts in either the presence or absence of KLH so that there were no net counts in the central system. Thus this assay permitted a direct correlation between colour development at 30 min and the actual amount of labelled mouse Ig (MIg) adhering to KLH-containing wells.

Adaptation of ELISA plates to cell culture

Experiments were carried out to determine whether KLH- or SRBC-immune spleen cells would secrete sufficient antibody to be detectable on ELISA plates when cultured overnight in antigen-containing wells. Spleens from mice to be tested (either primed and boosted or non-immune) were removed aseptically, teased apart in phosphate buffered saline (PBS) containing 5% foetal calf serum (FCS) (Flow Laboratories). The cell clumps were de-aggregated by expulsion through a tuberculin syringe with a 26 gauge needle. The cells were washed, counted directly using trypan blue exclusion, and the lymphoid cells were resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol and 50 μg per ml of gentamycin). Cells were dispensed in desired numbers into the ELISA microtitre wells labelled aseptically the day before with 0·2 ml of KLH at 10 μg per ml or 0·2 ml of SRBC soluble antigen at 10 μg per ml in carbonate buffer. Before the addition of cells to the wells, the plates were washed three times with sterile PBS. The final volume in each well was made up to 0·3 ml with complete medium and plates were incubated overnight at 37° in a humidified atmosphere of 5% CO_2. After overnight incubation the wells were emptied and the plates were developed as for the standard serological ELISA.

In vitro culture of cells

In order to determine the validity of this assay for the detection of antibody produced by cells cultured *in vitro* in the presence of KLH, spleen cells from KLH primed or non-immune mice were prepared as described above. Cells were cultured in 2·0 ml aliquots of complete medium at a concentration of 10^7 lymphoid cells per ml in the presence or absence of 1·0 μg/ml of KLH. Culturing was carried out in Linbro multi-well plates containing twenty-four flat bottom wells (No. 76–033–05) at 37° in a humidified incubator with 5% CO_2. Cells were harvested at various times, washed, counted, resuspended at the appropriate concentrations in complete medium and transferred to KLH-labelled ELISA plates for overnight culturing. The plates were developed as described above.

Plaque assay

Anti-SRBC plaque formation was assayed by the procedure of Cunningham & Szenberg (1968).

RESULTS

The quantification of the ELISA test used here was carried out using ^{125}I-labelled anti-KLH antibody from C57Bl/6 mice, and ^{125}I-labelled normal mouse Ig under equivalent conditions. The plates were developed colorimetrically, following which the individual wells were cut out and counted so that the actual levels of specific antibody adhering to the plates could be

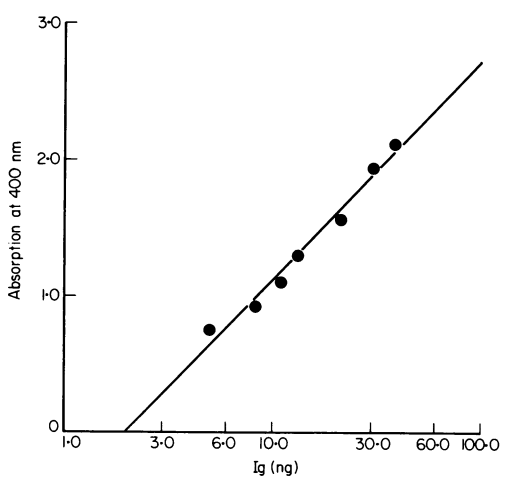

Figure 1. Relationship of ^{125}I-labelled anti-KLH Ig absorption to KLH (200 ng/ml) adhered to ELISA wells to development of colour with alkaline phosphatase labelled RAMIg. Plates were developed for 30 min before being read for absorbance at 400 nm. Net counts and readings were calculated by subtracting readings and counts found in preparations of ^{125}I-labelled Ig from equivalent tests with no antigen. The specific activity was $2{\cdot}2 \times 10^7$ c.p.m. per mg protein. SEM in each case was $< 1{\cdot}0\%$ and is not shown on the graph.

determined. The results are shown in Fig. 1. It can be seen that a linear relationship existed between the radioactivity detected and colour development. These results are based on mean data from two separate experiments, where the variation for individual data points between tests did not exceed ± 1·0%. Under the conditions used here, the colorimetric test was capable of accurately detecting mouse immunoglobulin (Ig) at levels below those detected by the radioimmunoassay. The colorimetric measurements are most sensitive between the spectrophotometic range of 0·1 and 0·8 and indicate levels of Ig below 10 ng. The radioassay was most reliable for levels at Ig above 10 ng, however, as the net counts for amounts of Ig below this level were less than 100. It was observed that levels of Ig below 2·0 ng per well could not be detected by either method when a 30 min incubation time was used to develop the plates. The labelled normal material used as a control in these tests gave slightly increased colour development at the two lowest dilutions tested, but otherwise were at background levels. The same radioactivity was detected with normal material whether or not KLH was adherent to the plates, so that net counts obtained with anti-KLH were attributed to specific antibody.

In order to determine the relationship between length of incubation and the degree of colour development, anti-KLH serum at 1:400 was tested in ELISA plates over 120 min. The results are shown in Fig. 2. As can be seen, net readings increased logarithmically

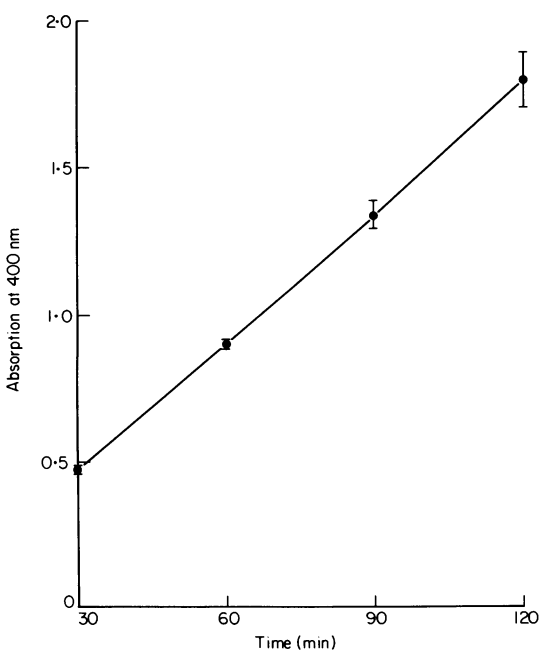

Figure 2. Colour development in relation to time in ELISA plates containing KLH (200 ng per ml) and anti-KLH (1:400). Data are presented as averages of four individual wells ± SEM. Antigen controls with antiserum at this dilution, even after 2 h incubation did not give readings above background reagents against which the spectrophotometer was zeroed.

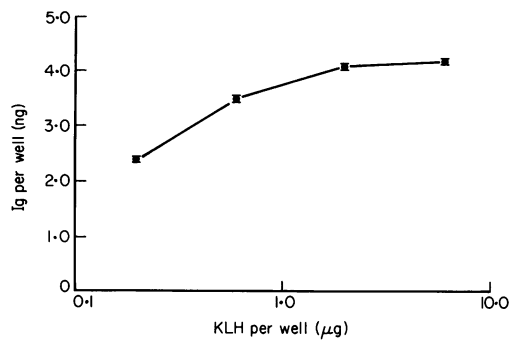

Figure 3. Titration of antigen (KLH) per well in detecting anti-KLH antibody produced in 18 h by immune splenocytes at 10^6 cells per well. Ig concentration was calculated by applying absorbance readings of wells developed for 30 min to the dose response curve shown in Fig. 1. Data are presented as averages of four individual wells ± SEM. Controls using non-immune cells or immune cells in the absence of KLH gave background level results.

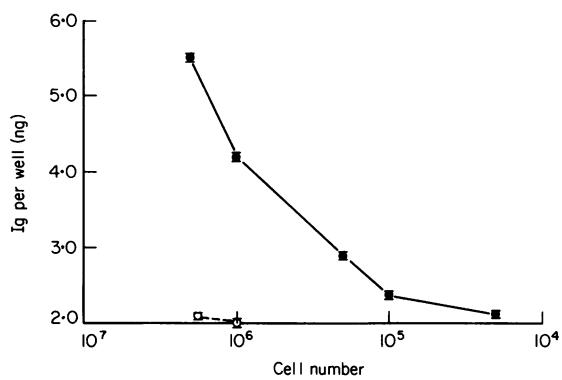

Figure 4. Titration of cell numbers in relation to ng anti-KLH detected. Immune splenocytes were incubated for 18 h at varying cell numbers per well in wells to which KLH had been attached at a concentration of 10·0 μg per ml. Immune splenocytes (•) yielded detectable Ig down to concentrations of 3×10^5. Non-immune (○) splenocytes gave barely discernable colour development at 3×16^6, otherwise control cultures were at background levels. Data are presented as averages of four individual wells ± SEM.

over this time period. Readings of controls also increased slightly during this time but the differential between controls and tests increased more, as shown in the figure which indicates the net increase. Thus, it is possible to extrapolate quantitative data from plates which have undergone prolonged incubation. It was found that prolonged incubation was necessary in order to develop significant levels of colour in some of the assays reported here.

The applicability of this procedure for use in estimating the production of antibody by cell populations was tested by incubating cells from the spleens of KLH-primed and boosted C57Bl/6 mice at 10^6 cells per well overnight in ELISA wells containing various concentrations of applied KLH. Appropriate controls were run simultaneously. Results are shown in Fig. 3. While good levels of anti-KLH antibody were detected between 0·6 and 6·0 μg KLH per 0·2 ml, only minimal amounts were detectable after 30 min colour development at 200 ng per well (the level optimal for serological testing). These data showed that it is possible to detect specific antibody being produced by primed cells using this technique. Further experiments involving cells were run on plates to which 2·0 μg of antigen had been fixed.

The numbers of immune spleen cells necessary for the detection of antibody by ELISA were tested by titrating cells on KLH plates between 3×10^6 and 3×10^4 cells per well. Cells were incubated overnight on ELISA plates and the plates were developed for 30 min. Results are shown in Fig. 4. Appreciable levels of antibody were detected under these conditions when cell numbers down to 3×10^5 per well were used in the assay.

It was clear that the ELISA procedure could be applied without difficulty to the measurement of specific antibody produced by splenocytes from immunized animals. Background levels of the various controls in this system never interfered with the specific response. A further series of experiments were undertaken to determine whether this technique was sensitive enough to detect antibody produced by primed cells cultured *in vitro* with KLH. Pooled populations of spleen cells from primed animals and controls were cultured over

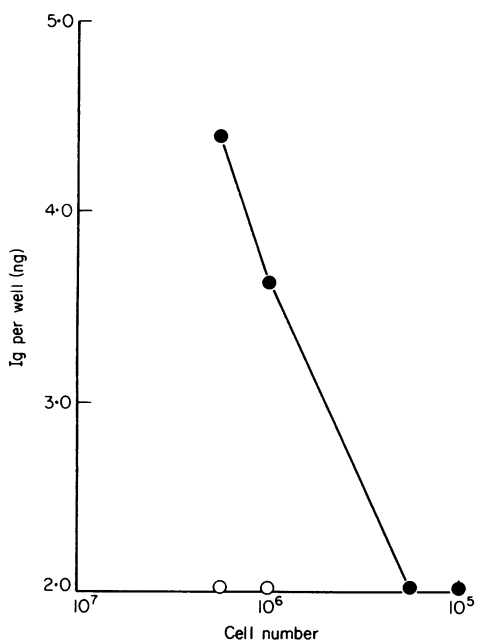

Figure 5. Detection of anti-KLH from cells subjected to a secondary *in vitro* antigenic stimulus. Spleen cells from KLH primed or non-immune animals were cultured for 4 days in the presence or absence of KLH. Cells were harvested and applied in varying numbers to KLH ELISA plates for 18 h after which the plates were developed. Only at concentrations of $2{\cdot}5 \times 10^6$ immune cells per well (●) were appreciable levels of anti-KLH detected. Controls (○), while showing the presence of some colour after 2 h development, had levels of Ig at or below the 2·0 ng level. Data points represent averages of four individual tests. SEM in these instances was < 1·0% and are not shown.

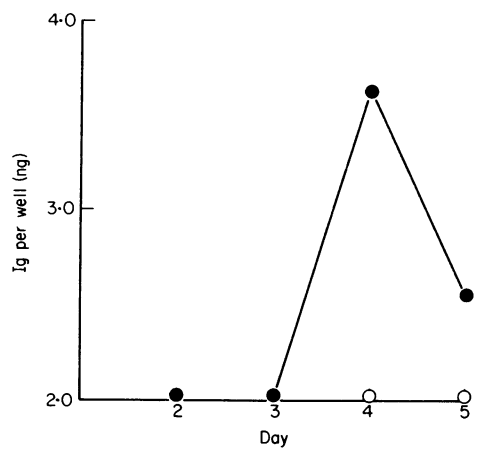

Figure 6. Detection of anti-KLH from 10^6 primed spleen cells (●) at various times after *in vitro* culture in the presence of KLH. Only on days 4 and 5 after culture initiation were appreciable levels of Ig detected. Other times of immune cell culture and cells from controls (○) showed the presence of some colour after 2 h development but levels of Ig were well below the 2·0 ng level, except the day 3 immune cell culture in which 1·8 ng were detected. Data points represent averages of four individual tests. SEM in these instances was < 1·0% and are not shown.

Table 1. Results obtained from secondary *in vitro* culture of KLH-primed or unprimed cells from individual animals in the presence or absence of KLH. After *in vitro* culture, cells were harvested and cultured overnight on ELISA plates. Tests were developed for 2 h. Absorbance readings were adjusted to the 30 min standard for calculating the ng Ig detected. Results represent averages from four non-immune and five KLH primed animals ± SEM.

Source of cells	*In vitro* culture	400 nm absorbance at 2 h		ng Ig detected per well†	
		10^6 cells*	3×10^5 cells	10^6 cells	3×10^5 cells
KLH-primed	+KLH	1·236 ± 0·205	0·619 ± 0·010	2·95 ± 0·49	1·20 ± 0·02
	no KLH	0·487 ± 0·054	0·296 ± 0·029	1·00 ± 0·11	0·75 ± 0·07
Non-immune	+KLH	0·566 ± 0·118	0·345 ± 0·059	1·08 ± 0·23	0·83 ± 0·14
	no KLH	0·572 ± 0·110	0·327 ± 0·052	1·13 ± 0·22	0·80 ± 0·13

* No of cells per well

† 2 h readings were used to calculate ngIg from the standard curve in Fig. 1. These values were divided by 4 on the basis of the data in Fig. 2 which shows a linear relationship between time and optical density.

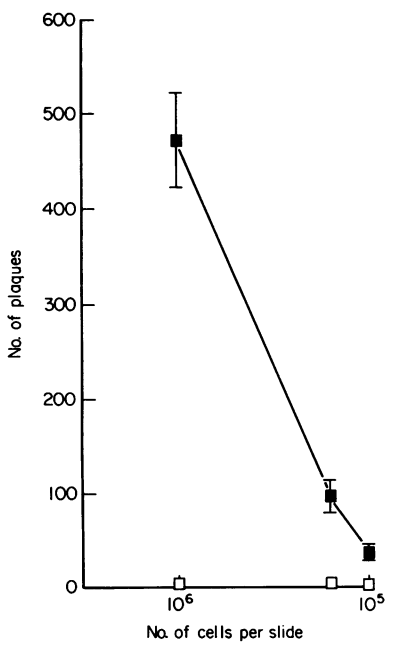

Figure 7. Numbers of plaques formed by varying numbers of SRBC-immune and non-immune spleen cells from CBA/J mice, ■, SRBC-immune; □, non-immune. Each data point represents the average ± SEM from duplicate assays run on six individual animals.

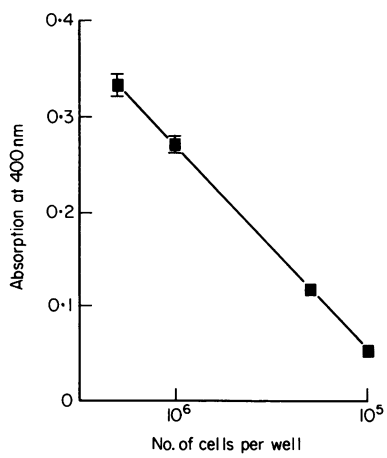

Figure 8. Detection of anti-SRBC antibodies produced by varying numbers of SRBC-immune spleen cells incubated for 18 h in microtitre wells to which SRBC soluble antigen had been attached. The data are presented as net readings from which readings obtained from comparable cultures of non-immune cells have been subtracted. Each data point represents the average of triplicate cultures from six individual immune animals ± SEM. SEM is not shown at 3×10^5 and 10^5 cells because it was < 0·02.

2–5 days in the presence or absence of KLH. On the various days, cells were harvested, washed and cultured in complete medium for a further 24 h on ELISA plates with and without KLH. The results are shown in Figs 5 and 6. It was found, that when cells were taken from the *in vitro* system it was necessary to develop the plates for longer than 30 min to obtain reasonable colour development in many instances. The results shown here are from plates incubated for 2 h. Readings made at that time were used to determine ng Ig per well, and then divided to determine the detectable

levels at 30 min (using the data from Fig. 2) in order to present all data on the same basis. In Fig. 5 it can be seen that cells taken after 4 days of culture with KLH produce detectable levels of antibody when cultured at $2{\cdot}5 \times 10^6$ and 10^6 cells per well on ELISA plates. All controls gave essentially background levels. The time course study (Fig. 6) carried out with 10^6 cells per well showed that detectable antibody was produced by cells cultured *in vitro* with KLH for 4 and 5 days. Again, control cultures gave results at background levels (Fig. 6).

In order to determine individual animal variation from the *in vitro* secondary response, cells from individual immunized and control animals were cultured for 4 days and subsequently tested at 10^6 and 3×10^5 cells per well on KLH ELISA plates. The results are summarized in Table 1 and show that while some variation between individuals is apparent, responses of immunized animals were obviously much higher than those observed with the controls. In this instance the KLH plates were developed for 2 h so that calculations of ng Ig were possible, even in instances where levels of Ig were lower than 2·0 ng per well. Again, absorbance readings were adjusted to the 30 min standard for calculating ng Ig. Since accuracy of such determinations has not yet been clarified, only amounts exceeding 2·0 ng Ig per well can be considered reliable.

It was of interest to compare the sensitivity of the ELISA procedure with the standard plaque assay. CBA/J mice, immunized with SRBC, were killed and their spleen cells used in both the plaque assay and the ELISA assay at various cell numbers. The results are shown in Figs 7 and 8. It can be seen that in both assays, antibody production was detectable down to the level of 10^5 immune spleen cells and, therefore, appear to be of comparable sensitivity.

DISCUSSION

The results reported here indicate that ELISA may have considerable application to the study of the antibody response to soluble antigens at the cellular level. Although this procedure is already widely used for the detection of anti-viral or parasitic antibody, there are only a few publications available on the actual quantitation of the technique. The combined radioimmunoassay and ELISA test carried out here show that a direct relationship exists between the amount of MIg detected on the antigen labelled plates and the degree of colour development in the enzyme assay at 400 nm. It is, therefore, possible to relate colour development to actual quantities of Ig attached to microtitre wells. This quantification was found to be reproducible to $\pm$ 1%. Under the conditions used here, the ELISA was more sensitive than the radioassay in that the greatest accuracy of colour development at 30 min was between 3·0 and 10·0 ng whereas the radio assay was most sensitive in the range of 10·0–30·0 ng (Fig. 1). Of course, this would not necessarily hold with the use of labelled Ig of higher specific activity or with highly labelled, purified specific Ig. Our finding that antibody is detectable in the range of 2·0 ng is in close agreement with findings published by others (Butler, Feldbash, McGivern & Stewart, 1978). It is realized that an assumption has been made that the specific activity of the anti-KLH antibody is analogous to that of whole mouse Ig, and that the accuracy of our conversion to nanograms of antibody from these data is contingent upon the specific activities of the whole Ig and the specific antibody being the same. The fact that our findings correlate so closely with those of others who have used different radioimmunoassay procedures to quantify ELISA data (Butler *et al.*, 1978) have led us to conclude that any error incurred by basing our calculations on the above mentioned assumptions, are probably not significant.

Our observation that there was a logarithmic relationship between absorbance at 400 nm and time of the colour development of tests over a period of 120 min has also been reported by others (Gripenberg, Linder, Kirki & Engvall, 1978). It is, therefore, possible to relate data from prolonged incubations back to standard curves established for 30 min. This may be important in developing tests in which very little antibody is being produced. Under the conditions used here, non-specific colour development in controls was not pronounced although background levels did increase slightly.

It was clear from our observations that spleen cells taken from KLH primed and boosted C57Bl/6 mice produce detectable levels of antibody when cultured overnight on ELISA plates to which KLH had been adsorbed (Fig. 3). Detectable levels were present when cell numbers were as low as 3×10^5 per well. Controls of either non-immune cells plus KLH or immune cells without KLH gave very low spectrophotometric readings indicating the specificity of the Ig detected. The data shown in this report all result from cell cultures incubated for 18 h in ELISA plates. If cell culture is continued for 48 or 72 h, levels of Ig on the plates are markedly increased while apparent specificity is main-

tained (unpublished data). Therefore, the sensitivity of this test may be increased by prolonged incubation.

The final experiments with KLH reported here involved the testing of primed cells which had been cultured *in vitro* with KLH for various times. Again, it was clear that such cells, when transferred at various times to ELISA plates containing KLH and incubated for a further 18 h produced sufficient antibody to be detectable in the assay under the conditions outlined above. As with cells taken directly from control animals, control cultures or those from short term cultures gave absorbance readings well below the levels observed from cultures producing specific antibody. Standard errors in all these tests were exceedingly small and the reproducibility of data within small limits of error was complete.

A comparison was made of the relative sensitivities of the plaque assay and antibody production in ELISA plates by SRBC immune spleen cells by setting up both assays with spleen cells of individual immunized and non-immunized animals. The data showed that the sensitivities of both techniques were similar, although the deviation between individual animals was considerably less using the ELISA technique (see error bars for Figs 7 and 8).

From further experiments underway in this laboratory, it is clear that the ELISA is easily applicable for use with a variety of other soluble antigens. It is also clear, however, that optimal levels for antigen concentration on the ELISA plates, and the actual detectable levels of Ig for individual systems differ with both the antigen under study and the species or strain from which the antiserum is derived. It is, therefore, essential to work out these parameters for a given system before applying this method in a quantitative manner for the detection of antibody being produced *in vitro* or *in vivo* by immune cells.

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