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Further studies of immunoglobulin synthesis by guinea-pig leukaemic lymphocytes

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Summary. The L_2C leukaemia is a B-lymphocytic neoplasm of strain 2 guinea-pigs, maintained by passaging *in vivo*. It synthesizes μ and λ immunoglobulin chains. These combine to form monomeric (7S) IgM molecules which are inserted into the plasma membrane. From here they are shed as monomeric IgM and as a species of higher molecular weight which has not been further defined. The synthesis of λ chain is in excess of that required for the IgM molecule, the surplus being exported directly from the cell without any intervening phase in the plasma membrane. Quantitative estimates of synthetic rates and pool sizes for these immunoglobulin species are presented.

INTRODUCTION

The L_2C tumour is a B-lymphocytic leukaemia of strain 2 guinea-pigs, maintained by passaging *in vivo* for 25 years (Nadel, Liu & Burstein, 1974; Shevach, Ellman, Davie & Green, 1972). We have previously reported the presence on the cells of surface IgM, its cleavage *in situ* by papain, and a demonstration of its idiotypic determinants (Stevenson, Eady, Hough, Jurd & Stevenson, 1975; Stevenson & Stevenson, 1975). The IgM, preserving the same idiotypic determinants, has been found on all sublines of the tumour in which it has been sought (Forni, Shevach & Green, 1976). A case for immunological attack on these determinants as a therapeutic measure has been presented (Stevenson, Elliott & Stevenson, 1977). An examination of the urine of leukaemia-bearing animals revealed the presence of a λ light chain apparently identical to that occurring in the leukaemic surface IgM (Stevenson, Mole, Raymont & Stevenson, 1975): it appears that the urinary protein represents a spillover due to a surplus synthesis, analogous to the Bence Jones proteins of human myeloma (Putnam & Miyake, 1958).

Here we report further studies of the assembly, display and export of immunoglobulin (Ig) by L_2C leukaemic lymphocytes. The pattern revealed might well apply to many B-lymphocytic neoplasms of man.

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MATERIALS AND METHODS

Immunoglobulins and antisera

The preparation of various guinea-pig Igs and Ig fragments has been described in previous publications (F. K. Stevenson *et al.*, 1975; G. T. Stevenson *et al.*, 1975). IgG, Fab'γ and light (L) chains were prepared from normal guinea-pig serum, while κ chains were prepared from purified anti-dinitrophenyl antibody where their concentration is greatly enhanced (Nussenzweig, Lamm & Benacerraf,

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1966). Guinea-pig λ chains were prepared either from normal guinea-pig L chains (normal λ chain), by immunosorptive removal of the κ chains, or from the urine of animals in the terminal phase of the L_2C leukaemia (L_2C λ chain).

A new method was used for the isolation of IgM from guinea-pig serum. This method combines gel-filtration and immunosorption and results in a product which is free from contamination with non-Ig macroglobulins. Guinea-pig serum was fractionated on Ultrogel AcA 34 (LKB) to obtain a '19S fraction' which contained the IgM. A column of Sepharose 4B coupled to IgG from sheep anti-guinea-pig L chain by the CNBr method (Porath, Axen & Ernback, 1967) was used to isolate Ig from the 19S fraction, the volume of the anti-L column being just sufficient to bind all the Ig present. Bound Ig was eluted from the column with 0·5 M NH_4OH and immediately dialysed against 1 M tris buffer, pH 8·0. Ig eluted from the anti-L column was next passed through a column of Sepharose 4B-anti-sheep IgG, followed by a final separation on Ultrogel AcA 34. IgM was eluted in a position corresponding to the original '19S fraction'. IgM prepared by this method and examined by immunoelectrophoresis using anti-guinea-pig whole serum showed a single arc in the expected position for IgM.

Monomeric (7S) IgM was prepared by subjecting the whole molecule to reduction with 1 mM dithiothreitol at room temperature for 15 min, alkylating with iodoacetamide (in a 10% molar excess of SH groups) at room temperature for 30 min, and isolating the 7S IgM by chromatography on AcA 34. Fabμ was prepared by papain digestion of serum IgM as described previously (G. T. Stevenson *et al.*, 1975) but with digestion time increased to 18 h.

Antisera to guinea-pig Igs were raised in sheep or rabbits as described by G. T. Stevenson *et al.* (1975). Antiserum to Fab'γ gave good precipitin reactions with both IgG and IgM and was used as a polyspecific anti-guinea-pig Ig serum. Anti-Fabμ was made specific for Fdμ determinants by absorption with guinea-pig IgG coupled to Sepharose 4B. Precipitin analysis showed that the resultant anti-Fdμ reacted with IgM but not with IgG, Fab'γ or L chain. Antiserum to normal guinea-pig λ chain was used without further absorption.

Leukaemic cells and cellular fractions

Cells were obtained from leukaemia-bearing animals and washed as described previously (G. T. Stevenson *et al.*, 1975). Limited proteolysis of the cell surfaces by papain (Eady, Hough, Kilshaw & Stevenson, 1974) was carried out by exposing the cells at 10^8/ml in Eagle's minimal essential medium (MEM) to papain (0·06 mg/ml) for 30 min at 37° in the presence of bovine pancreatic deoxyribonuclease (0·2 mg/ml); digestion was terminated by alkylation with iodoacetamide. Cell lysates were prepared by suspending washed cell pellets at 10^8/ml for 30 min in phosphate-buffered saline, pH 7·4 (PBS) containing 1% Nonidet P40 (British Drug House), Trasylol (Baeyer) 500-KIU/ml, and iodoacetamide (0·2 M).

Immunofluorescence

The direct technique for immunofluorescent staining of the surface Ig of L_2C cells has been described by G. T. Stevenson *et al.* (1975). For indirect staining, cells in suspension (2×10^7 cells/ml) were treated for 30 min with an equal volume of IgG solution (1 ml/ml) prepared either from the appropriate antiserum or from normal serum. The cells were washed three times with PBS at 4°, resuspended in a small volume of PBS and treated with fluorescein conjugated anti-Ig for 30 min at 4°. Stained cells were washed and the percentage of fluorescent cells scored as previously described.

Cell surface iodination

Cell surface proteins were labelled with ^{125}I using the lactoperoxidase-catalysed iodination technique as modified by Haustein (1975). The labelled cells were washed twice in PBS at 4° and lysed as described above. The lysate was centrifuged at 4° for 30 min at 4000 *g* and the supernatant passed through a column of Sephadex G25 to remove any labelled material of low molecular weight.

Specific immune precipitation

Igs were specifically precipitated from cell lysates or culture supernatants by the direct method. Aliquots of solution containing labelled Ig were mixed with guinea-pig IgG (5 μg) and rabbit anti-guinea-pig Ig serum (100 μl). This volume of antiserum had been shown to be sufficient to precipitate all the carrier IgG. A non-specific control precipitation was set up containing human IgG (5 μg) and rabbit anti-human γ chain serum (100 μl). After standing 24 h at 4° the precipitates were sedimented and washed three times in PBS at 4°.

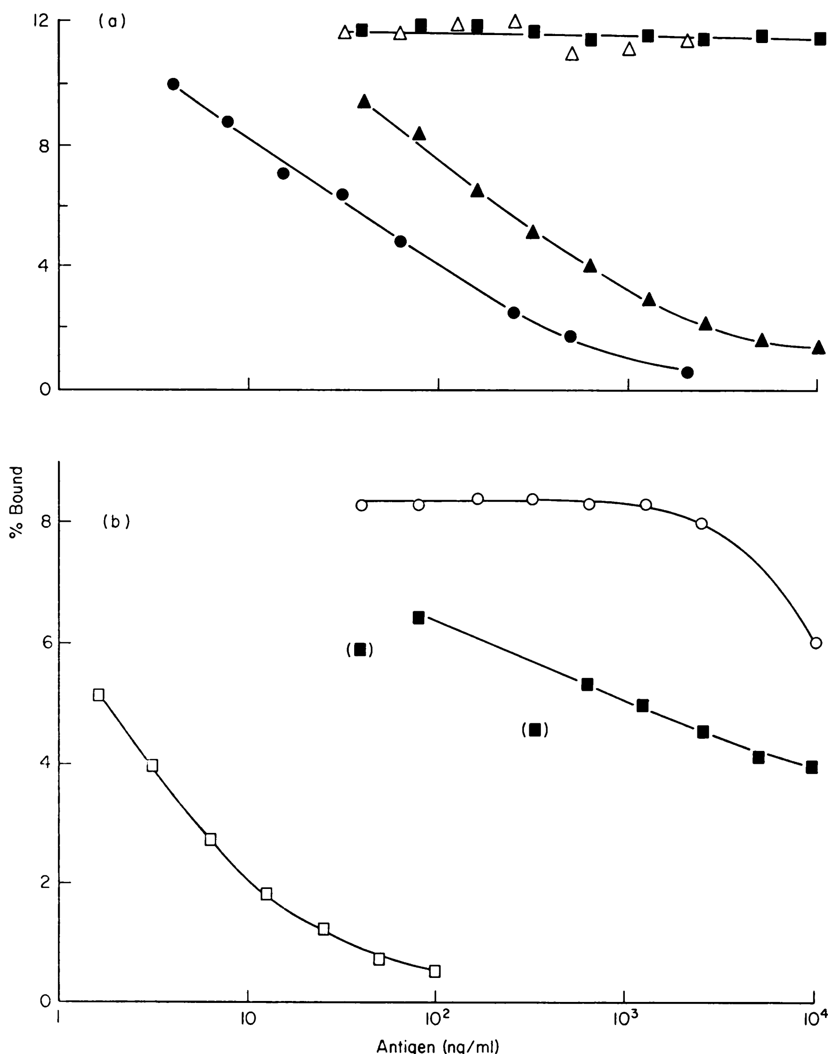

Figure 1. Radioimmunoassay for guinea-pig Fabμ (a) and guinea-pig λ chain (b). Curves show the inhibition of binding of labelled antigen to solid-phase antibody in the presence of unlabelled Fabμ (●), 7S IgM (▲), IgG (■), L chain (△), L_2C chain (□) and κ chain (○). Each point represents the mean of triplicate determinations which, apart from the bracketed values, agreed within $\pm 6\%$. (a) Fabμ assay. The antibody is rabbit anti-guinea-pig Fdμ coupled to Sephadex G-25, the labelled antigen ^{125}I-Fabμ. (b) λ chain assay. The antibody is sheep anti-guinea-pig λ chain coupled to Sephadex G-25, the labelled antigen ^{125}I-L_2C λ chain.

Polyacrylamide gel electrophoresis

Specific immune precipitates were dissolved, reduced, alkylated and analysed by electrophoresis on 7·5% polyacrylamide gels containing 0·1% sodium dodecyl sulphate (SDS-polyacrylamide; Vitetta, Baur & Uhr, 1971). Purified human IgG and IgM were labelled with ^{125}I by the chloramine T method (Hunter, 1967), treated as above, and run as markers on parallel gels. After electrophoresis for 3–5 h at 80 V, gels were cut into 2 mm slices and counted to determine their ^{125}I or ^{14}C contents.

Radioimmunoassays

Solid phase radioimmunoassays for Ig antigens have been described by Eady, Chapple, Hough & Stevenson (1975). Using a method similar to that described

we have now obtained assays specific for Fabμ and λ chain. The Fabμ assay used anti-Fdμ coupled to Sephadex G25 Superfine (Pharmacia) as solid phase with Fabμ as labelled antigen. For the λ chain assay, anti-normal λ chain was coupled to the solid phase with L_2C chain as labelled antigen.

Fig. 1a demonstrates the specificity of the Fabμ assay. The assay was capable of quantifying Fabμ at concentrations in the range 0·5–100 ng/ml. The response curve for 7S IgM was parallel to the curve for Fabμ and, as observed in the analogous system for the assay of human Fabμ (Eady *et al.*, 1975), the response curve was displaced with respect to the Fabμ curve in a way which might suggest that only a fraction of the Fabμ determinants in the intact IgM molecule are able to react simultaneously with the solid phase antibody. Cross reactivity with neither IgG nor L chain could be detected in the Fabμ assay at concentrations up to 1000 ng/ml.

Fig. 1b shows the specificity curves for the λ chain assay. The assay showed no cross reactivity with κ chain at concentrations up to 1000 ng/ml, while IgG showed partial cross reactivity. The fact that the response curve for IgG was not parallel to the λ chain curve, together with the apparent inability of IgG to displace all labelled λ chain from the solid phase at high IgG concentrations, suggests that the λ chain assay was responding in part to determinants expressed only on free λ chain (Epstein & Gross, 1964).

RESULTS

Ig on plasma membrane

The distribution of surface Ig was examined by indirect immunofluorescence, using rabbit anti-Fdμ followed by fluorescein-conjugated sheep anti-rabbit IgG. When the entire procedure was carried out at 4°, 90–95% of cells in the L_2C preparation showed strong circumferential staining with superimposed bright spots. If the exposure to anti-Fdμ was at 37°, followed by chilling and staining with fluorescein-anti-rabbit IgG at 4°, few (< 10%) cells stained and these showed the staining in caps.

When cells treated with anti-Fdμ at 37° were stained for residual membrane Ig using fluorescein conjugated anti-guinea-pig Ig, only occasional cells (< 10%) were stained. These cells showed stained caps; no cells were detected with circumferential staining. All the surface Ig appears therefore to have reacted with anti-Fdμ.

To characterize the Ig chains accessible to iodination on the plasma membrane the L_2C surfaces were labelled with ^{125}I, and Ig was subsequently isolated from cell lysate by immune precipitation. After subtraction of the control precipitation value, anti-Ig was seen to have precipitated 1·4% of the total ^{125}I in the lysate. When the labelled precipitate was dissolved, reduced, alkylated and analysed by electrophoresis on SDS-polyacrylamide gel, two peaks of labelled material were detected (Fig. 2). The peaks corresponded to the positions of μ chain and L chain markers. The Ig isolated from labelled lysate gave a slightly higher ratio of L chain counts to μ chain counts than was seen with a labelled IgM standard.

Ig released during cell culture or upon treatment with papain

Ig released from L_2C cells was estimated using the radioimmunoassays specific for Fabμ and λ chain. Table 1 lists typical results for cells cultured in MEM and for cells exposed to papain under non-toxic conditions.

The Ig in simple culture fluid is thought to represent IgM released metabolically from the cell surface, plus free λ chains exported directly from the cytoplasm; there is also likely to be a contribution from trauma sustained by the cells during manipulations.

Papain appears to cleave surface IgM *in situ* on the plasma membrane to release Fabμ-like fragments (G. T. Stevenson *et al.*, 1975). On this basis the assays in Table 1 for papain digest of L_2C cells are thought to reflect the presence of Fabμ from surface IgM cleaved *in situ*, Fabμ from metabolically released IgM, and exported λ chains; clearly the first two species contribute to the Fabμ assay and all three to the λ assay. The Fabμ from surface IgM appears as the difference between supernatants from digest and culture. It has varied over a two-fold range, has consistently exceeded 50% of the Fabμ obtainable by lysis of the cells with 1% Nonidet P40, and exceeds earlier estimates based on a less specific assay (G. T. Stevenson *et al.*, 1975). The figures in Table 1 suggest that approximately 60,000 Fabμ molecules per cell have been released from the surface IgM by papain.

Following the finding of a λ Bence Jones protein in the urine of guinea-pigs in the terminal phase of L_2C leukaemia (F. K. Stevenson *et al.*, 1975) it was of interest to assay supernatants of L_2C cultures for

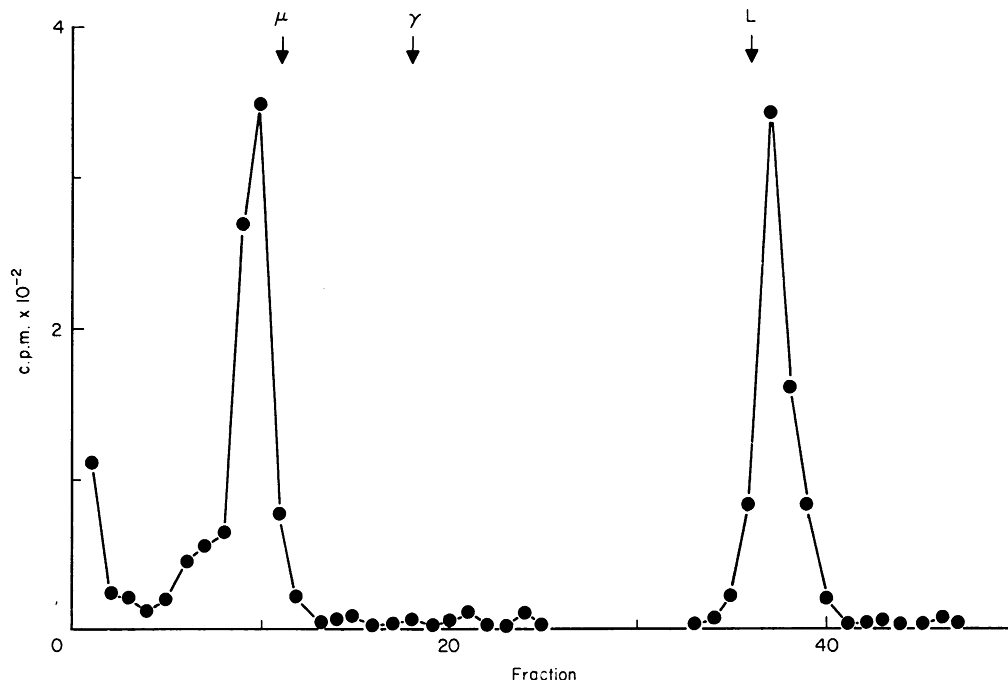

Figure 2. Electrophoretic analysis in SDS-polyacrylamide gel of membrane Ig from iodinated L_2C cells. The membrane proteins were labelled with ^{125}I by the lactoperoxidase method. Ig was isolated from cell lysate by precipitation with anti-guinea-pig Ig. The immune precipitate was washed, solubilized, reduced and alkylated prior to electrophoretic analysis on 7·5% SDS-polyacrylamide gel. After electrophoresis for 3·5 h at 80 V, the gel was cut into 2 mm slices for counting. ^{125}I-labelled marker proteins were run on parallel gels and the positions of marker peaks are indicated by arrows.

the accumulation of λ chain. Fig. 3 shows the results obtained when supernatants from cultures were assayed for Fabμ and λ chain contents. The λ chain contents appear much the greater but there is uncertainty about reactivities in the assays of the precise molecular species released from the cells. The rate of release of Ig is seen to have fallen gradually over a 6 h period; data for longer periods are vitiated by poor cell survival after 6 h at 37°.

Intracellular Ig

Cell lysates revealed by radioimmunoassay approximately 80 ng of Fabμ and 40 ng of λ chain per 10^7

Table 1. Radioimmunoassay of Ig in L_2C cell supernatants

Treatment of cells	Radioimmunoassay for: Fabμ (ng/10^7 cells)	Radioimmunoassay for: λ chain (ng/10^7 cells)
Culture only, 30 min, 37°	15	15
Papain at 0·06 mg/ml, 30 min, 37°	64	26

L_2C cells at 2×10^7/ml in MEM were incubated at 37° with or without papain. After 30 min the papain digest was alkylated so as to inactivate the enzyme. The supernatant from the enzyme-free culture, after separation from the cells, was subjected to digestion by papain so as to yield similar molecular forms of Ig for comparison by the assays. All values were corrected by subtraction of a reagent blank.

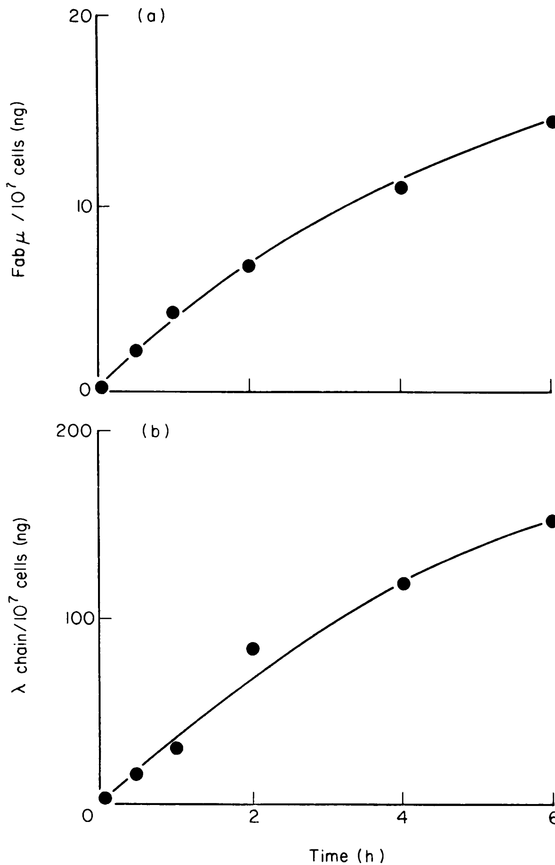

Figure 3. Radioimmunoassays of L_2C cell culture supernatants. L_2C cells were cultured at 1×10^7/ml in Eagles MEM. Cells were sedimented and the supernatants assayed for reactivities in radioimmunoassays for (a) Fabμ, (b) λ chain.

cells, the figures varying over a two-fold range in different experiments. In one experiment Ig in the lysate was subjected to digestion by papain (0·06 mg/ml for 30 min at 37°, with the Nonidet diluted out to 0·1%); the assayed amount of Fabμ then increased by a factor of 1·4, consistent with some IgM having been cleaved to release fragments more active in the assay (see Fig. 1a).

The synthesis and nature of intracellular Ig were further studied by culturing the cells for 8 h at 10^7/ml with ^{14}C-leucine (58 Ci/mmol, 2 μCi/ml) in leucine-free MEM. After subtracting control values anti-Ig was found to precipitate 4·4% of the macromolecular ^{14}C from the cell lysate and 25% from the culture supernatant, the macromolecular ^{14}C being taken as that precipitable by trichloroacetic acid.

The nature of the labelled Ig in this experiment was investigated by electrophoresis in SDS-polyacrylamide of the reduced and solubilized immune precipitates (Fig. 4). The patterns were complex. The cell lysate (Fig. 4a) revealed two peaks (1 and 3) in positions consistent with μ and λ chains respectively. The largest peak between these (peak 2) corresponds to material frequently observed in this position (e.g. Vitetta, McWilliams, Phillips-Quagliata, Lamm & Uhr, 1975; Gordon, Hough, Karpas & Smith, 1977) and thought by some to be related to the surface Fcγ-receptor. The cell supernatant (Fig. 4b) revealed a peak in the position of the L chain marker, superimposed upon a considerable irregular background.

Molecular sizes of released and intracellular Ig

Having established that L_2C cell supernatants from simple culture, papain digestion and Nonidet P40 lysis all contain both Fabμ- and λ chain-related material, it was important to distinguish between free λ chain and the λ chain component of Fabμ or intact IgM. This was achieved by gel filtration of L_2C cell supernatants on Ultrogel AcA 44. The elution of Ig was monitored by radioimmunoassay of the fractions for both Fabμ and λ chain. Fig. 5 compares the elution profiles obtained with papain digest supernatant (Fig. 5a), 37° culture supernatant (Fig. 5b) and Nonidet P40 lysate (Fig. 5c). The method is clearly able to resolve free λ chain and Fabμ-related material with a λ chain component.

The radioimmunoassay profile of digest supernatant shows an Fabμ-related species containing λ chain, followed by a peak of free λ chain. The Fabμ-related material moves faster on the gel than does Fabμ prepared from human serum IgM, an anomaly we are investigating. The culture supernatant reveals mainly free λ chain, with a small amount of leading Fabμ-related material. The cell lysate reveals Fabμ-related and free λ peaks of similar sizes.

The Fabμ-related materials from both culture supernatant and lysate, moving slightly faster on AcA 44 than the Fabμ-related material from digest, were in positions consistent with whole IgM, either monomeric (7S), pentameric (19S), or monomeric with attached membrane lipid (Vitetta & Uhr,

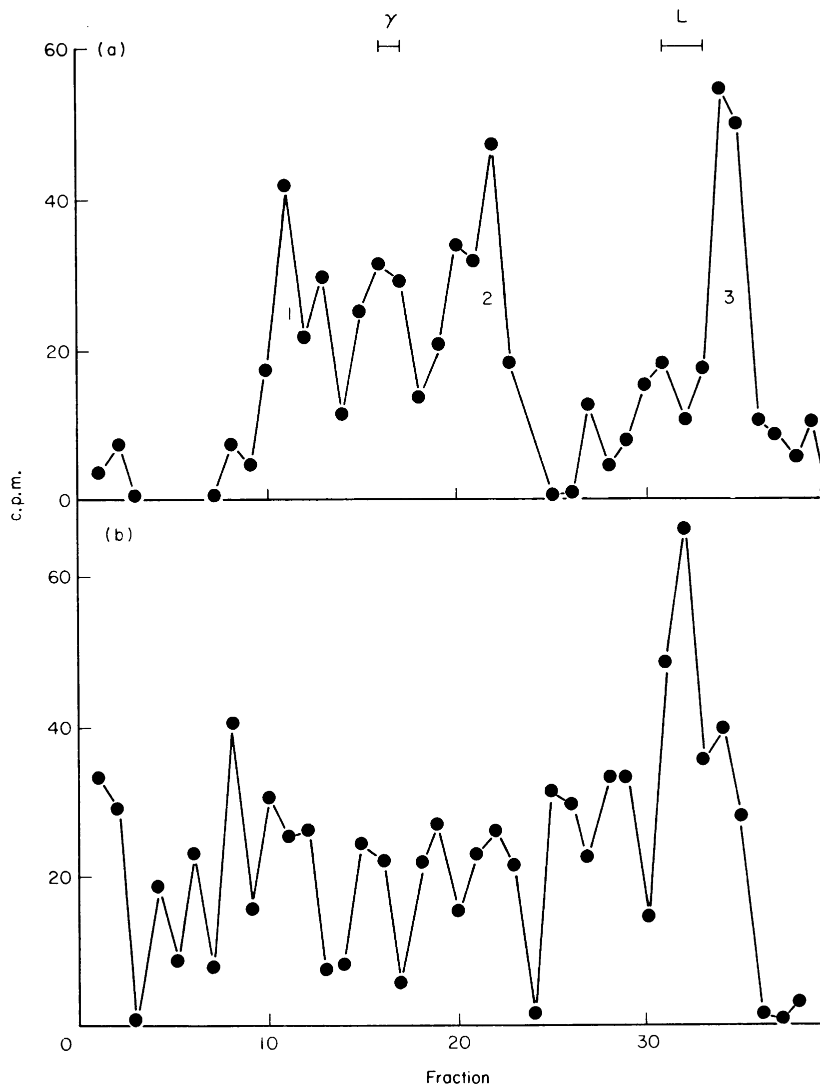

Figure 4. Electrophoretic analyses in SDS-polyacrylamide gel of Ig from L_2C cells cultured with ^{14}C-leucine. The markers apply to both analyses. (a) Cell lysate. Peak 1 is in a position consistent with μ chain and peak 3 with λ chain. (b) Culture supernatant. The largest peak is consistent with λ chain.

1972). When culture supernatant was chromatographed on the more porous gel AcA 34 (Fig. 6) most Fabμ activity appeared in the eluate close to 150,000 suggesting monomeric IgM. However there was also an appreciable amount of activity in a peak of higher molecular weight. Conclusions regarding the relative concentrations of these two Fabμ-containing species cannot be drawn from the peak areas because we do not know their activities per unit mass in the assay.

Urinary λ chain

Guinea-pigs bearing a large load of L_2C leukaemia, for example some 12 days after inoculation and 2 days before death, reveal a homogeneous band, of

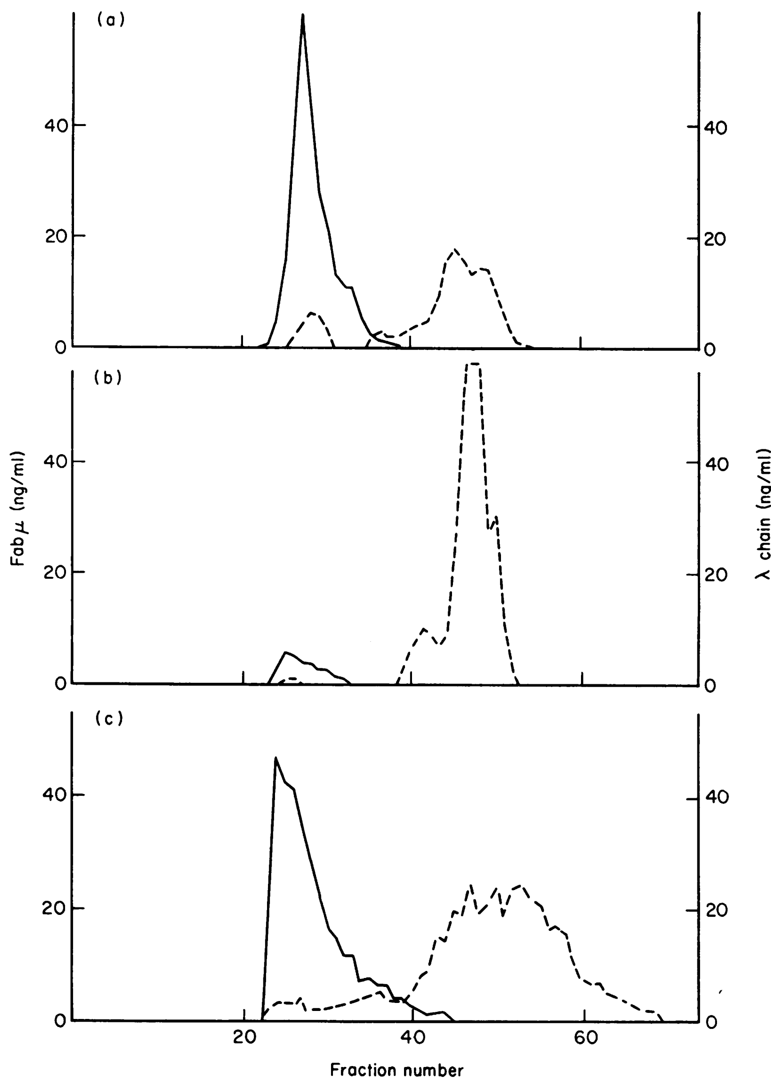

Figure 5. Analysis of L_2C cell supernatants by gel filtration and radioimmunoassays. Nonidet P40 lysate (1×10^8 cells/ml lysed in PBS containing 1% Nonidet P40), and ten times concentrated papain digest (1×10^8 cells/ml digested with 0·06 mg/ml papain) or cell culture supernatant (1×10^7 cells/ml cultured 8 h in Eagles MEM) were chromatographed on Ultrogel AcA 44 ($1{\cdot}6 \times 85$ cm) equilibrated with PBS. Fractions were 2·8 ml. Aliquots from each were assayed for Fabμ (——) and λ chain (– – –); (a) digest, (b) culture, (c) lysate. N.B. The chain assay reacts differently with free and combined λ chains (see Methods and Fig. 1). Thus a given amount of λ chain will not give the same peak height when present in free form as in combination with a heavy chain.

mobility between α and β, in urinary concentrate examined by a standard clinical electrophoretic method. The separation of this protein, its identification as a λ chain by amino acid sequence, its rate of excretion as a function of tumour load, and its apparent identity with the light chain of the cell surface IgM have been reported previously (F. K. Stevenson *et al.*, 1975; Stevenson, Elliott & Stevenson, 1977).

When examined in the analytical centrifuge the λ chain from urine revealed a single symmetrical peak with $S_{20,w} = 3{\cdot}2$ at 3 mg/ml. This is consistent with the chains existing predominantly or entirely as dimers. The dimers appear to be stabilized by

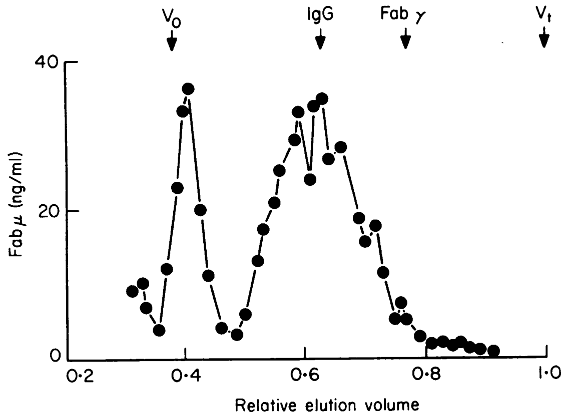

Figure 6. Analysis of the supernatant of an L_2C culture by gel filtration and radioimmunoassay for Fabμ. One ml of a 30-fold concentrate was applied to a column of AcA 34, 1·6 × 58 cm, equilibrated with PBS. The *x*-axis shows elution volume relative to total column volume.

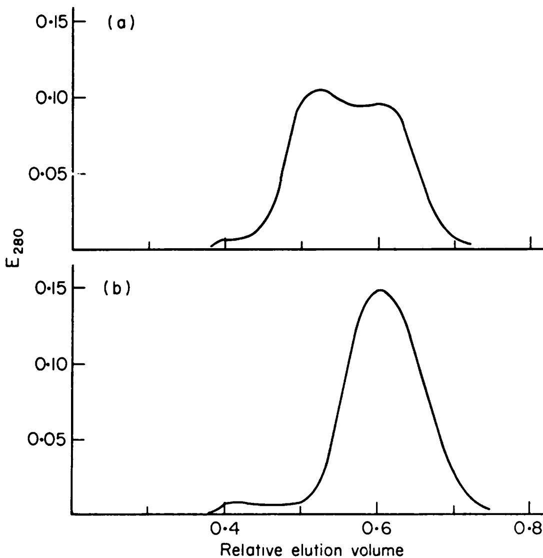

Figure 7. Gel filtration of urinary λ chain, unreduced (a) or reduced (b), on Sephadex G-100 equilibrated with 1 M propionic acid. In each case 2 ml of protein at 1·6 mg/ml was loaded onto a column 1·8 × 86 cm, the runs being conducted at room temperature. The *x*-axis shows elution volume relative to total column volume. The sample for run (b) was first reduced with dithiothreitol (10 mM, 10 min, room temperature) and then alkylated with iodoacetamide (22 mM, 30 min, room temperature).

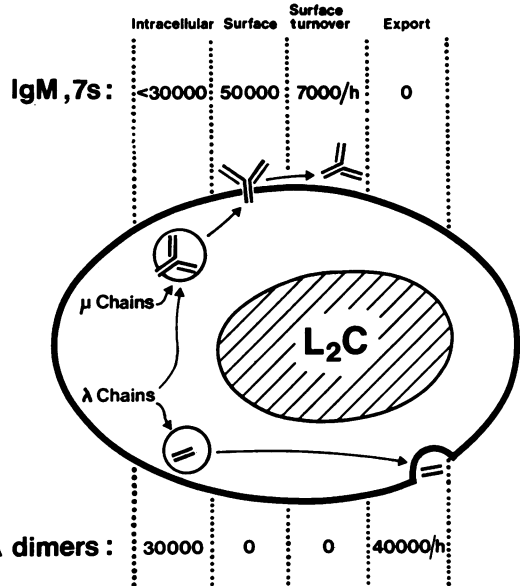

Figure 8. A summary of Ig production by L_2C leukaemic lymphocytes.

noncovalent bonds and in some molecules by disulphide bonds as well; this is analogous to what has been observed with human κ Bence Jones proteins (Milstein, 1965). Thus when subjected to gel chromatography in 1 M propionic acid, conditions which disrupt noncovalent bonds, the chain reveals a double peak consistent with dimer and monomer (Fig. 7a). However, when the same procedure is carried out on a sample previously reduced and alkylated to sever interchain disulphide bonds, only one peak is seen in the monomeric position (Fig. 7b).

DISCUSSION

Our present concepts of the production of Ig by L_2C leukaemic lymphocytes are summarized in Fig. 8. The uniform reactivities of the cells with anti-Ig reagents and their morphological uniformity, in particular the lack of plasmacytoid forms, suggest that there is no significant range of differentiation with implied variations in Ig production. The numbers, derived from data in this and a previous paper (G. T. Stevenson *et al.*, 1975) are offered only as a rough guide. They involve various compromises. For example the suggested number of surface IgM molecules is more than would be suspected

from the yield of Fabμ in digests but less than suggested by the uptake of radiolabelled anti-Ig onto the surface.

It will be seen that the term 'export' is applied conventionally to material delivered to the extracellular environment, presumably from membrane-bound vesicles, without an intervening surface phase. The fact that antibody specific for μ chains caps all surface Ig reinforces our earlier evidence against the presence of free light chains on the surface membrane, in contrast to a report for human cells (Fu, Winchester & Kunkel, 1973); it also makes it unlikely that any IgD, a class not yet described for guinea-pigs—is on the surface. The rate of accumulation of IgM in the cellular supernatants is adequately accounted for by the turnover on the plasma membrane (G. T. Stevenson *et al.*, 1975) so it is unlikely that any significant quantity of whole IgM follows the export path.

L_2C surface IgM appears to be in the monomeric or 7S state (Konen, Green, Shevach, Kask & Schwartz, 1976), as has been found for mouse splenic lymphocytes (Vitetta, Baur & Uhr, 1971). The results in Fig. 6 indicate that it is released from the membrane as a mixture of monomeric and larger forms. The nature of the latter is not known; for mouse splenic lymphocytes the release of IgM with some attached membrane lipid has been suggested (Vitetta & Uhr, 1972).

The L_2C cell is clearly a useful tool for studying many aspects of guinea-pig Ig synthesis. Surface IgM, with apparently identical idiotypes, was present in all five sublines of the tumour examined by Forni *et al.* (1976), even in the presence of variable expression of Ia and tumour transplantation antigens. The Ig synthesis appears therefore to be an ancestral feature persisting through the vicissitudes of passaging for 25 years. The λ chain appearing in the urine of tumour-bearing animals is the only monoclonal Ig from the guinea-pig now readily available for structural studies.

Ig synthesis by the L_2C cell is also a valuable indicator of events likely to be occurring in B-lymphocytic neoplasms of man. We refer especially to the non-exporting neoplasms, where whole Ig molecules are assembled simply for surface display and where in consequence the idiotypic determinants of these molecules are an attractive therapeutic target (Stevenson & Stevenson, 1975). The export of surplus light chains from the cell invalidates neither the definition of non-exporting nor the target value of idiotypic determinants on whole molecules (G. T. Stevenson *et al.*, 1977). As regards cell surface chemistry the L_2C appears an immaculate B cell, with its surface displaying in addition to Ig a complement receptor, an Fcγ receptor, and Ia antigen (Shevach *et al.*, 1972; G. T. Stevenson *et al.*, 1975; Forni *et al.*, 1976). There is already some indication that the striking surplus synthesis of light chains found in L_2C cells is common among the non-exporting B neoplasms of man (Maino, Kurnick, Kubo & Grey, 1977; Gordon & Smith, personal communication). Studies of Ig synthesis by these neoplasms might help in classifying them.

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