Endocytosis and subsequent processing of ^{125}I -labelled immunoglobulin G by guinea pig yolk sac *in vitro*

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(Received 19 November 1984/Accepted 26 November 1984)

We have developed conditions for studying the binding, uptake, degradation and transport of ^{125}I -labelled IgG by yolk sac *in vitro*. Specific binding to tissue at 4°C and to paraformaldehyde-treated tissue at 37°C was time- and temperature-dependent and showed saturation kinetics ($K_{d,4^\circ\text{C}} = 2.9 \times 10^{-6}\text{ M}$, $K_{d,37^\circ\text{C}} = 5.3 \times 10^{-6}\text{ M}$). Uptake was studied at 37°C using untreated tissue ($K_{\text{uptake}} = 13.3 \times 10^{-6}\text{ M}$) and was inhibited by preincubation with metabolic poisons but not with cycloheximide. Tissue that had been incubated with ^{125}I -labelled IgG at 37°C released radiolabelled degradation products and intact ^{125}I -labelled IgG into the medium. Experiments with paraformaldehyde-treated and untreated tissue showed that release of intact ^{125}I -labelled IgG was mostly the result of ligand dissociation from surface binding sites. However, more ^{125}I -labelled IgG was released from untreated tissue than could be accounted for solely by loss of surface-bound ligand and the difference was presumed to reflect uptake, transport and exocytosis of ^{125}I -labelled IgG. Degradation of ^{125}I -labelled IgG was inhibited by leupeptin and lysosomotropic amines. These drugs had no detectable effect on ^{125}I -labelled IgG release. The results suggest that degradation and transport of IgG are not intimately related and are consistent with a previously proposed model for IgG transport via coated vesicles which do not fuse with lysosomes and for non-selective uptake into another class of vesicle which does fuse with lysosomes.

Receptor-mediated endocytosis is one of the processes by which cells take up macromolecules from the external environment (Anderson & Kaplan, 1983). In many instances internalized ligand is eventually delivered to the lysosomal system and degraded. Other systems in which receptor-mediated endocytosis is believed to be involved, but which have received comparatively less attention, are those where a macromolecule is internalized, translocated within the cell and then released intact into some other extracellular compartment. Examples include the transport of IgA and other serum proteins from blood to bile via hepatocytes (Jones *et al.*, 1982) and the transmission of maternal immunoglobulin to the fetus (Rees & Wallace, 1980). In the latter case, the route and timing of transmission varies depending on species. In the rabbit and the guinea pig, maternal IgG is transported to the fetus during the last half of gestation via endodermal cells of the visceral yolk sac (Brambell, 1970; Wild, 1970; King & Enders, 1970) and there is good evidence that

transport is mediated by cell surface receptors which recognize the Fc region of IgG (Brambell *et al.*, 1960; Kaplan *et al.*, 1965; Wild, 1979). Wild (1975, 1976) and King (1977, 1982) have proposed that IgG binds to receptors which are associated with, or which become associated with, clathrin-coated pits on the apical surface of yolk sac endodermal cells. These pits are envisaged as giving rise to coated vesicles which do not fuse with lysosomes but which move to, and fuse with, the basolateral plasma membrane, whereupon the intact IgG is exocytosed. Degradation is thought to result from non-selective uptake of protein into another class of vesicle which does fuse with lysosomes.

Previous reports from this laboratory have been concerned with describing the cytological events involved in the uptake and transport of IgG by the guinea pig yolk sac (King & Enders, 1970; King, 1977, 1982). However, in order to obtain quantitative information on this process and to enable us to study the mechanism of transport, we have

investigated the uptake and processing of ^{125}I -labelled IgG by the guinea pig yolk sac *in vitro*, and it is these results which are reported here.

Experimental

Chemicals

Unless stated otherwise, all incubations were carried out in Eagle's Minimum Essential Medium (Gibco Laboratories, Grand Island, NY, U.S.A.) containing globulin-free bovine serum albumin (Sigma Chemical Co.) at a final concentration of 10 mg/ml. The solution was adjusted to pH 7.3 after addition of the albumin and was gassed with a mixture of O_2/CO_2 (19:1). Leupeptin and pepstatin were purchased from Sigma and dissolved in dimethyl sulphoxide prior to dilution with aqueous medium. NaCN, NaF, 2,4-dinitrophenol, chloroquine and cycloheximide were obtained from Sigma and paraformaldehyde from Fisher Scientific Co., Santa Clara, CA, U.S.A. Guinea pig IgG was purchased from Miles Laboratories Inc., Elkhart, IN, U.S.A. Na^{125}I and $[\text{U}-^{14}\text{C}]\text{sucrose}$ (584 mCi/mmol) were obtained from the Amer-sham Corporation, Arlington Heights IL, U.S.A.

Iodination

IgG was iodinated with Na^{125}I and 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodo-Gen; Pierce Chemical Co., Rockford, IL, U.S.A.) (Fraker & Speck, 1978; Beisiegel *et al.*, 1981). Unreacted Na^{125}I was removed by passing the reaction mixture down a small column (0.5 ml bed volume) of Dowex 1X8-100. The iodinated product was stored in 100 μl aliquots at -20°C and used within 2 weeks. Just before use, the material was passed down a Sephadex G-200 column and any radioactivity which appeared in the void volume was discarded. The major peak of radioactivity, which was identified as monomeric IgG by comparison with the elution properties of standard proteins of known M_r , was collected and concentrated as necessary by vacuum dialysis. More than 98% of the radioactivity was precipitable in 10% (w/v) trichloroacetic acid and the specific radioactivity was usually between 80 and 200 c.p.m./ng of protein.

Yolk sac preparation

Yolk sacs were removed from diethyl ether anaesthetized 55-day pregnant guinea pigs, care being taken to avoid non-villous tissue and amnion. The tissue was placed immediately in medium and incubated in a water bath at 37°C for 45 min. During this preincubation period the tissue was washed three times over a nylon mesh and reincubated each time in fresh medium. This procedure was undertaken in order to allow the

clearance of endogenous IgG from surface binding sites. The tissue was then cut into small pieces (2 mm $\times$ 5 mm; 2–6 mg wet wt.) which were washed again over nylon mesh and used immediately for experiments.

Binding and uptake studies

Only the basic experimental protocol will be given here. Details unique to a particular experiment are given in the appropriate Figure and Table legends. For binding and uptake studies pieces of yolk sac were incubated individually and without agitation in medium (0.25 ml) containing ^{125}I -labelled IgG. For the determination of non-specific binding, parallel incubations were set up containing ^{125}I -labelled IgG and, where possible, a 100-fold excess of unlabelled guinea pig IgG. At the end of the incubation, the samples were filtered over a nylon mesh and the retained tissue was washed with 3 ml of ice-cold, radioactive isotope-free medium. After a further 10 min incubation at 4°C in medium (2 ml), the tissue was again washed over a nylon mesh, blotted dry, weighed and then placed in a γ -radiation counter for determination of radioactivity. Specific binding/uptake is defined as total binding/uptake (i.e. radioactivity associated with tissue in the absence of excess unlabelled IgG) minus non-specific binding/uptake (i.e. radioactivity associated with tissue in the presence of excess unlabelled IgG).

The low temperature washing procedure just described was designed to allow the removal of unbound or non-specifically associated radioactivity with minimal loss of receptor-bound ligand. Non-specifically associated radioactivity is an inherent problem with this system and, even using the washing protocol just described, accounted for between 40 and 60% of total tissue-associated counts. The ratio of specific to non-specific binding could be increased slightly by extending the wash period to 20 min, but the recovery of specifically bound radioactivity began to decline under these conditions, presumably due to dissociation of ligand from surface receptors.

Degradation and release studies

For these experiments tissue was incubated with ^{125}I -labelled IgG as described above. For some experiments, excess unlabelled IgG was also present. After washing at 4°C to remove unbound radioactivity, the tissue pieces were reincubated individually in radioactive isotope-free medium (1 ml). At the appropriate time, samples (0.5 ml) of incubation medium were removed and treated with 0.5 ml of 20% (w/v) trichloroacetic acid. After 30 min at 4°C , the samples were centrifuged for 2 min at 15600 g in an Eppendorf 5414 microcentrifuge and the resulting supernatant (acid-soluble

fraction) was removed and counted for radioactivity. The pellet (acid-insoluble fraction) was washed with ice-cold 10% (w/v) trichloroacetic acid and then counted for radioactivity. The tissue pieces were washed over a nylon mesh with ice-cold medium (2ml), blotted, weighed and finally counted for radioactivity.

Paraformaldehyde treatment of yolk sac

Yolk sac was preincubated at 37°C for 45 min and small pieces were prepared as described above. The tissue pieces were treated with a solution of 1% (w/v) paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4°C for 30 min. Paraformaldehyde was removed by washing the tissue with medium for 30 min at 37°C, with four changes of medium. The washed, treated tissue pieces were then used immediately for experiments.

Measurement of tissue ATP levels

Pieces of yolk sac (2–6 mg wet wt.) were quickly frozen in liquid N₂ and, where processing was not done immediately, the tissue was stored under liquid N₂ for up to 2 weeks. Frozen tissue was hand-homogenized with 0.3 ml of 6% (w/v) HClO₄ in 20% (v/v) ethanol (pre-cooled to –15°C) and left to stand on ice for 30 min. The homogenate was centrifuged at 15 600g for 30 s and the supernatant was removed and neutralized by the addition of 2M-KOH (0.2 ml). The precipitated KClO₄ was removed by centrifugation and the supernatant was used immediately for determination of ATP or frozen and stored in liquid N₂ until needed.

Tissue extracts were suitably diluted (usually 500-fold) with 0.1 M-Tris/acetate buffer, pH 7.7, containing 2 mM-EDTA, and assayed for ATP by a modified luciferin–luciferase method using the ATP-Monitoring Kit (LKB Instruments, Gaithersburg, MD, U.S.A.) and following the manufacturer's instructions. The final reaction volume was 1 ml. Luminescence was measured using a Searle Analytic 92 liquid-scintillation counter with preset count and time at 800 000 and 0.5 min, respectively, and a Channel A window of 60–65 divisions.

Measurement of [¹⁴C]sucrose uptake

Pieces of yolk sac were prepared as described above and incubated with [¹⁴C]sucrose at 37°C. Concentrations and incubation times are given in the figure legends. The tissue pieces were then washed exactly as described for IgG. After washing and weighing, the tissue pieces were solubilized by the addition of 0.5 ml of a mixture (9:1, v/v) of NCS solubilizer (Amersham Corporation) and water and incubation at 45°C for 4 h. PCS scintillation fluid (10 ml) (Amersham Corporation) was then added and the samples counted for

radioactivity in a Searle Analytic 92 scintillation counter. The washing procedure was judged to be effective on the basis of the following. Tissue was exposed to [¹⁴C]sucrose (20 μM) at time zero (actual elapsed time in contact with tracer was approximately 4 s) and tissue-associated radioactivity was then measured both before and after washing. More than 92% of the initial tissue-associated radioactivity was removed by the procedure.

Determination of endocytic index

Endocytic index is defined as the volume of medium whose contained substrate is endocytosed per unit time and per unit tissue (Williams *et al.*, 1975). For sucrose, the endocytic index was obtained directly from the slope of the uptake/rate graph. For IgG, values were calculated from the uptake/rate graph after correction for ligand degradation. The endocytic index for receptor-mediated IgG uptake was calculated from the slope of the initial linear portion of the specific uptake/rate graph. Obtained in this way, values were expressed relative to wet weight of tissue. These values were then re-expressed relative to tissue protein on the basis that 1 mg of tissue contains 0.18 mg of protein. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Treatment of results

Where appropriate, data have been expressed as means ± s.d. for the number of experiments indicated, each experiment representing data from a different animal. Within an experiment all determinations were carried out at least in triplicate. Curves through data points have been fitted by eye, exceptions being Scatchard and double-reciprocal plots where lines of best fit were obtained by linear regression analysis.

Results

Binding of ¹²⁵I-labelled IgG

Binding was first examined at 4°C and a time course study revealed that an equilibrium was reached in 6 h (Fig. 1a). Using a 6 h incubation time, we studied the concentration-dependence of specific binding and found that the system became saturated at ¹²⁵I-labelled IgG concentrations greater than 0.25 mg/ml (Fig. 1b). At saturation, about 0.4 pmol of IgG were bound/mg of tissue. When the specific binding data were replotted by the method of Scatchard (1949) an apparent equilibrium dissociation constant (*K*_d) of 2.9 × 10^{–6} M was obtained. Non-specific binding increased linearly with concentration with no indication of saturability. This may reflect the presence of low-affinity, high-capacity non-

Fig. 1. Binding of ^{125}I -labelled IgG to yolk sac

(a) Time-course of binding. Pieces of yolk sac were incubated with ^{125}I -labelled IgG (0.1 mg/ml) at 4°C for the times indicated, then washed and counted for radioactivity (see the text for details). (b) Concentration-dependence of binding. Pieces of yolk sac were incubated with the indicated amounts of ^{125}I -labelled IgG for 6 h at 4°C . (c) and (d) Concentration-dependence of binding to paraformaldehyde-treated yolk sac at 37°C and 4°C , respectively. The incubation time was 6 h. Specific binding was determined as described in the text. The insets to (b), (c) and (d) show Scatchard plots of the specific binding data. Results are mean values for three experiments in each case.

specific binding sites. It could also result from the inability of the washing procedure to remove soluble tracer from the tissue adequately. The latter seems less likely, however, since washing effectively removed the fluid-phase marker [^{14}C]sucrose (see the Experimental section). Furthermore, it was previously found (King, 1982) that exposure of yolk sac to ferritin (a high- M_r ,

fluid-phase marker) at 4°C , followed by washing, resulted in no detectable tissue-associated ferritin, as determined by electron microscopy.

Measurement of IgG binding at 37°C is complicated by virtue of the fact that ligand internalization also occurs at this temperature. We chose to overcome this problem by pretreating tissue with paraformaldehyde, which inhibits most meta-

bolic processes, including endocytosis (Rosenblith *et al.*, 1973; Kaplan & Nielsen, 1979; Tietze *et al.*, 1982) and which, therefore, facilitates binding studies at 37°C (Kaplan & Nielsen, 1979; Tietze *et al.*, 1982; Tsay & Schlamowitz, 1975). Acceptance of this approach as valid requires that paraformaldehyde treatment does not alter the ligand-binding properties of cell surface receptors or result in permeabilization of the plasma membrane such that ligand can diffuse into cells and bind to intracellular sites. An adverse effect on surface receptors is unlikely since binding of ^{125}I -labelled IgG to untreated and treated yolk sac at 4°C was the same (Figs. 1b and 1d). Furthermore, experiments in which paraformaldehyde-treated yolk sac was exposed to horseradish peroxidase-conjugated IgG for 1 h at 37°C and then examined by electron microscopy revealed reaction product only in coated pits associated with the microvillous plasma membrane. In contrast to untreated tissue, no reaction product was found in intracellular structures (results not shown). These observations support the use of paraformaldehyde-treated yolk sac for studying surface ligand-receptor reactions.

The concentration-dependence of binding at 37°C to treated yolk sac is shown in Fig. 1(c) and the apparent equilibrium dissociation constant was calculated to be $5.3 \times 10^{-6} \text{ M}$. From the data it was estimated that 0.4–0.5 pmol of IgG would be bound at saturation.

Uptake of ^{125}I -labelled IgG

These experiments were carried out with untreated yolk sac at 37°C in order to allow both the binding and internalization of added ligand. The time course study presented in Fig. 2(a) shows that increasing amounts of radioactivity became associated with the tissue during the first 30 min of incubation, but that an apparent equilibrium was reached by about 60 min. A possible explanation for this observation is that yolk sac gradually loses viability during incubation at 37°C. However, this idea was rendered unlikely by the following observations. Firstly, yolk sac which was incubat-

Fig. 2. Uptake of ^{125}I -labelled IgG (a) Time course of uptake. Pieces of yolk sac were incubated with ^{125}I -labelled IgG (0.1 mg/ml) at 37°C for the times indicated, then washed and counted for radioactivity (●). ○ Shows uptake after correction for the loss of degradation products into the medium; △ shows specific uptake. (b) and (c) shows the concentration-dependence of total ^{125}I -labelled IgG uptake and specific ^{125}I -labelled IgG uptake, respectively. The incubation time was 90 min. The inset shows a double-reciprocal plot of the specific uptake data. Results are mean values for three experiments in each case.

ed at 37°C for 1 h in the absence of ligand and then exposed to ligand at 37°C showed the same pattern of uptake as tissue exposed to ligand without prior incubation in ligand-free medium (results not shown). Secondly, ATP levels in yolk sac incubated for 2 h at 37°C were not significantly different from levels in freshly dissected tissue (see below). Thirdly, the uptake of [^{14}C]sucrose, a non-degradable fluid phase marker, increased linearly with time (Fig. 3a). A more likely explanation was suggested by analysis of the incubation medium surrounding yolk sac pieces, which was found to contain increasing amounts of acid-soluble radioactivity. This material was shown to consist of mono-iodotyrosine (see the next section) and is indicative of hydrolytic degradation of ^{125}I -labelled IgG. When radioactivity lost from the tissue as the result of degradation was accounted for, total uptake was seen to increase in a linear manner (Fig. 2a). The uptake of ^{125}I -labelled IgG was inhibited by a variety of metabolic poisons (Table 1). Cycloheximide, however, had no significant effect on uptake for up to 2 h. The same dose of cycloheximide inhibits yolk sac protein synthesis, as determined by the incorporation of [^{14}C]leucine into acid-insoluble material, by more than 95% within 20 min (results not shown). None of these drugs had any effect on ligand binding at 4°C (results not shown).

The concentration-dependence of uptake was studied by using a 90 min incubation time (Fig. 2b). Total uptake was measured up to a maximum ligand concentration of 25 mg/ml and evidence of an approaching plateau was obtained. Unfortunately, it was not possible to measure specific uptake over the same range because of the unacceptably large amounts of unlabelled IgG needed to determine non-specific uptake at ligand concentra-

tions greater than 1 mg/ml, and no indication of saturability was evident (Fig. 2c).

The uptake of [^{14}C]sucrose was also studied and found to be dependent on concentration over the range examined (Fig. 3b).

Table 2 shows endocytic indices for ^{125}I -labelled IgG and [^{14}C]sucrose calculated from the data in Figs. 2(a) and 3(a) as described by Williams *et al.* (1975) and Moore *et al.* (1977). The endocytic index for ^{125}I -labelled IgG was determined from the slope of the line after correction for substrate

Table 1. Effect of metabolic inhibitors on the uptake of ^{125}I -labelled IgG

Pieces of yolk sac were preincubated with or without drugs for 15 min at 37°C in glucose-free medium. ^{125}I -labelled IgG (0.1 mg/ml) was added and the incubation was continued for 2 h at 37°C after which the tissue was washed and the tissue-associated radioactivity was determined. Results are mean values ($\pm$ s.d.) from three experiments.

Treatment	Total uptake of ^{125}I -labelled IgG (ng/mg of tissue)
None	71.6 $\pm$ 10.1
2,4-Dinitrophenol (0.5 mM)	16.8 $\pm$ 2.9
NaF (10 mM)	11.6 $\pm$ 2.6
NaCN (5 mM)	13.3 $\pm$ 2.8
Cycloheximide (10 μg /ml)	68.4 $\pm$ 15.2

Fig. 3. Uptake of [^{14}C]sucrose by yolk sac (a) Time-course of uptake. Pieces of yolk sac were incubated with [^{14}C]sucrose (20 nmol/ml) for the times indicated, after which the tissue was washed and the tissue-associated radioactivity determined as described in the Experimental section. At time zero, tissue-associated radioactivity was 342 c.p.m./mg of tissue. (b) Concentration-dependence of uptake. The incubation time was 90 min. Each point is a mean value derived from two separate experiments.

Table 2. *Endocytic indices for [^{14}C]sucrose and ^{125}I -labelled IgG*

Rates of uptake were derived from Figs. 2(a) and 3(a) and were converted into endocytic indices as described in the Experimental section and in the text. Values are means ($\pm$ S.D.) for the number of experiments shown in parentheses.

Tracer	Rate of uptake (c.p.m./h per mg wet wt. of tissue)	Endocytic index ($\mu\text{l/h}$ per mg of tissue protein)
[^{14}C]Sucrose (20 nmol/ml, 1095 c.p.m./pmol)	3702(2)	0.95(2)
^{125}I -labelled IgG (0.1 mg/ml, 74 c.p.m./ng)		
Total	5170 $\pm$ 678(3)	3.91 $\pm$ 0.81(3)
Specific	2776 $\pm$ 427(3)	2.10 $\pm$ 0.51(3)

degradation. In the case of specifically taken up IgG, where uptake did not rise linearly with time (Fig. 2a), the value for the endocytic index was calculated from the slope of the line drawn through the first three time points. It should be noted that values for IgG do not take into account the loss of intact ligand resulting from exocytosis (see below) and may, therefore, be an underestimate.

Release of radioactivity from yolk sac

As noted above, yolk sac incubated continuously in the presence of ^{125}I -labelled IgG released acid-soluble radioactivity into the medium. When yolk sac was exposed to ^{125}I -labelled IgG at 37°C for 60 min, then washed and reincubated at 37°C in radioactive isotope-free medium, it was apparent that both acid-soluble and acid-insoluble radioactivity were released (Fig. 4). Additional characterization of the released radioactivity was achieved by applying samples of medium to a Bio-Gel P-6 column. About 40% of the radioactivity was co-eluted with standard mono-iodotyrosine and the remainder was eluted in the exclusion volume. Rechromatography of this high- M_r material on Sephadex G-200 resulted in the appearance of a single radioactive peak which was co-eluted with unlabelled, monomeric IgG (results not shown).

Experiments were conducted with the object of obtaining more information on these processes.

Degradation of ^{125}I -labelled IgG

No acid-soluble radioactivity was generated if ligand was incubated at 37°C in the absence of tissue or if ligand was incubated at 37°C with spent medium (results not shown), confirming that proteolysis is not mediated by secreted proteinases. The effect of compounds known to perturb the lysosomal system was investigated next. Since some of these compounds (NH_4Cl , chloroquine) also inhibit internalization and receptor recycling (Livesey *et al.*, 1980, Dean *et al.*, 1984) and may inhibit IgG receptor recycling in the guinea pig yolk sac (G. C. Douglas & B. F. King, unpublished work), tissue was exposed to the drugs after first

Fig. 4. *Release of radioactivity from yolk sacs previously exposed to ^{125}I -labelled IgG*

Pieces of yolk sac were incubated with ^{125}I -labelled IgG (0.1 mg/ml) for 90 min at 37°C , then washed and reincubated in radioactive isotope-free medium at 37°C (see the text for details). At the time indicated, samples were removed for determination of tissue-associated radioactivity (●), acid-insoluble radioactivity in the medium (▲) and acid-soluble radioactivity in the medium (○). Results are mean values from three experiments.

being allowed to take up ^{125}I -labelled IgG. The results (Table 3) show that in the presence of NH_4Cl and chloroquine, degradation was inhibited by 67% and 46%, respectively, and by 40% in the presence of the cysteine proteinase inhibitor, leupeptin. However, pepstatin, a specific inhibitor of cathepsin D, had no detectable effect on IgG degradation. In order to give the tissue an opportunity to attain greater intracellular concentrations of the cathepsin inhibitors, experiments were carried out in which tissue was preincubated with leupeptin and pepstatin for 30 min prior to addition of ligand, as described by Weisbecker *et al.* (1983). Under these conditions the inhibition of degradation seen with leupeptin was slightly

greater (47%) but, once again, no effect of pepstatin was found (results not shown).

Table 3 also shows that the release of acid-insoluble radioactivity was unaffected by any of the drugs tested.

Release of ^{125}I -labelled IgG

The release of intact ^{125}I -labelled IgG from tissue previously exposed to ligand can be explained by (1) internalization of IgG followed by intracellular transport and subsequent discharge (exocytosis) of ligand and/or (2) direct loss of ligand from non-specific or specific surface binding sites. In order to evaluate the extent to which ligand was being lost from surface binding sites, we studied the release of ^{125}I -labelled IgG from yolk sac which had been treated with paraformaldehyde. Paraformaldehyde-treated tissue is unable to

internalize ligand by endocytosis and is also impermeable to added IgG; thus any loss of ligand reflects desorption from the tissue surface. Fig. 5 shows that total release from treated tissue was similar to that from untreated tissue over the first 30 min of incubation, but by 60 min release from the treated tissue had levelled off while release from untreated tissue continued to rise.

These results indicated that as much as 80% of

Table 3. Effects of proteinase inhibitors and lysosomotropic agents on the degradation of ^{125}I -labelled IgG

Pieces of yolk sac were incubated with ^{125}I -labelled IgG (0.1 mg/ml) for 90 min at 37°C, then washed and reincubated in radioactive isotope-free medium for 90 min under the conditions indicated. The temperature was 37°C unless stated otherwise. Acid-soluble and acid-insoluble radioactivity in the medium were determined as described in the text. Results are mean values ($\pm$ S.D.) for three experiments and are expressed relatively by taking the control value to be 100%.

Treatment	Relative release of radioactivity (% of control)	
	Acid-soluble	Acid-insoluble
Leupeptin (50 $\mu\text{g}/\text{ml}$)	60 $\pm$ 12	98 $\pm$ 19
Pepstatin (50 $\mu\text{g}/\text{ml}$)	102 $\pm$ 24	101 $\pm$ 21
NH_4Cl (10 mM)	33 $\pm$ 12	101 $\pm$ 24
Chloroquine (0.1 mM)	54 $\pm$ 15	91 $\pm$ 25
4°C	7.7 $\pm$ 3.1	27 $\pm$ 10

Fig. 5. Release of ^{125}I -labelled IgG from paraformaldehyde-treated and untreated yolk sac

Pieces of paraformaldehyde-treated (see the text for details) and untreated yolk sac were incubated with ^{125}I -labelled IgG (0.1 mg/ml) for 90 min at 37°C. The tissue pieces were washed and reincubated in radioactive isotope-free medium. At the times specified, samples were removed and the acid-insoluble radioactivity in the medium was determined. Results are mean values ($\pm$ S.D.) from three experiments.

Table 4. Release of ^{125}I -labelled IgG from paraformaldehyde-treated and untreated yolk sac

Pieces of paraformaldehyde-treated and untreated yolk sac were incubated with ^{125}I -labelled IgG (0.1 mg/ml) in the presence and absence of excess unlabelled IgG (10 mg/ml) for 90 min at 37°C. After washing, the tissue was reincubated in radioactive isotope-free medium for 90 min at 37°C or 4°C as indicated. The amount of acid-insoluble radioactivity in the medium was determined and non-specific release were calculated as described in the text. Results are mean values ($\pm$ S.D.) from five experiments. Statistical analysis was carried out using Student's *t* test; **P* < 0.05, ***P* < 0.001, N.S., not significant (*P* > 0.05).

Reincubation temperature	Tissue	Amount of acid-insoluble radioactivity released (ng/ml of tissue)		
		Total	Non-specific	Specific
37°C	Untreated	18.1 $\pm$ 2.6	11.4 $\pm$ 2.1	6.7 $\pm$ 0.9
	Treated	13.8 $\pm$ 2.1*	12.0 $\pm$ 1.7 N.S.	2.8 $\pm$ 1.1**
4°C	Untreated	3.3 $\pm$ 1.0	0.7 $\pm$ 0.4	4.0 $\pm$ 0.5
	Treated	3.2 $\pm$ 0.4 N.S.	0.8 $\pm$ 0.5 N.S.	3.6 $\pm$ 0.9 N.S.

the intact IgG released from untreated yolk sac after 90min could simply have dissociated from the tissue surface. We sought to determine, however, whether or not the remaining 20% could reflect IgG transport.

If receptor-mediated internalization and transport of IgG was contributing to the observed release of ligand, then prevention of the initial

binding of ligand to receptor should abolish transport and cause a reduction in the total release of intact IgG. The results in Table 4 show that if ¹²⁵I-labelled IgG was prevented from binding to receptors by the presence of excess unlabelled IgG during the first incubation, the amount of intact ligand subsequently released from untreated yolk sac was reduced and was similar to that released from treated tissue. Thus, the difference in the patterns of total release seen for treated and untreated tissue (Fig. 5) is due to the greater specific release from the latter. Table 4 also shows the effect of low temperature on ligand release. When the reincubation was carried out at 4°C, total ¹²⁵I-labelled IgG release was reduced by more than 75% in both treated and untreated yolk sac, showing that the major effect was to decrease the rate of ligand dissociation from the surfaces sites.

Fig. 6 shows the ligand concentration-dependence of specific ¹²⁵I-labelled IgG release from untreated yolk sac. The dissociation constant for the reaction was 10.7×10^{-6} M.

Uptake and processing of ¹²⁵I-labelled bovine IgG and bovine serum albumin

Both these proteins were taken up by yolk sac and, in pulse-chase experiments, 60% of the initial tissue-associated ¹²⁵I-labelled bovine serum albumin and 40% of the initial tissue-associated ¹²⁵I-labelled IgG was degraded within 90 min (Table 5). In both cases, intact protein was released into the medium, but, in contrast with homologous IgG, the amounts released from treated and untreated tissue were similar.

ATP levels in yolk sac

The level of ATP in freshly dissected yolk sac was 1.32 ± 0.14 nmol/mg of tissue. Yolk sac incubated in gassed medium at 37°C for 3h had a mean ATP concentration of 1.12 ± 0.16 nmol/mg of tissue. Tissue incubated in the presence of 2,4-

Fig. 6. Concentration-dependence of ¹²⁵I-labelled IgG release

Pieces of yolk sac were incubated with the indicated amounts of ¹²⁵I-labelled IgG for 90min at 37°C. After washing, the tissue was reincubated in radioactive isotope-free medium for 90 min at 37°C. Specific release of ¹²⁵I-labelled IgG is shown (see the text for definition and experimental details). The inset shows a double-reciprocal plot of the data. Results are mean values from three experiments.

Table 5. Degradation and release of ¹²⁵I-labelled bovine serum albumin and ¹²⁵I-labelled bovine IgG

Pieces of paraformaldehyde-treated and untreated yolk sac were incubated with either ¹²⁵I-labelled bovine serum albumin or ¹²⁵I-labelled bovine IgG (each at 0.1 mg/ml) for 90 min at 37°C, then washed and reincubated in radioactive isotope-free medium for 90 min at 37°C. The amount of acid-soluble and acid-insoluble radioactivity released into the medium was determined as described in the text. Results are mean values ($\pm$ s.d.) from the number of experiments shown in parentheses. Abbreviation used: N.D., none detectable.

¹²⁵ I-labelled protein	Tissue	Tissue-associated radioactivity at start of reincubation (c.p.m./mg of tissue)	Amount of radioactivity released into medium (c.p.m./mg of tissue)	
			Acid-soluble	Acid-insoluble
Bovine serum albumin	Untreated	3184 $\pm$ 586 (3)	1913 $\pm$ 478 (3)	809 $\pm$ 161 (3)
	Treated	1362 $\pm$ 274 (3)	N.D.	851 $\pm$ 188 (3)
Bovine IgG	Untreated	10488 (2)	4162 (2)	5126 (2)
	Treated	7700 (2)	N.D.	4976 (2)

dinitrophenol (10mM) for 90 min at 37°C had a mean ATP concentration of 0.25 ± 0.10 nmol/mg of tissue.

Discussion

Maternofetal transmission of IgG in the guinea pig occurs across the visceral yolk sac towards the end of the normal 68 day gestation period (Brambell, 1970) and previous reports from this laboratory have been concerned with characterizing the cytological events involved in this process (King & Enders, 1970; King, 1977, 1982). In the present paper we have quantified the binding, uptake, and degradation of ^{125}I -labelled IgG by the guinea pig yolk sac *in vitro*. Yolk sac viability under conditions *in vitro* was judged to be good on the basis that there was no detectable change in tissue ATP levels during normal incubation and that the uptake of [^{14}C]sucrose, an inert fluid-phase marker, proceeded in a linear manner with time. Furthermore, electron microscopic examination of yolk sac has shown that morphological integrity is maintained under standard incubation conditions.

Binding and uptake studies

^{125}I -labelled IgG binds to guinea pig yolk sac at 4°C and 37°C in a manner that is both specific and saturable, so providing evidence for the earlier suggestion (King, 1977, 1982) that guinea pig yolk sac bears cell surface receptors for IgG. Results from experiments *in vivo* on several IgG transporting systems are compatible with the idea that transport is a receptor-mediated process (see Brambell, 1970; Rodewald, 1980). Binding studies *in vitro* with the rabbit yolk sac have confirmed the presence of a specific IgG receptor with an apparent dissociation constant of 1.85×10^{-5} M (Tsay & Schlamowitz, 1975). The dissociation constant for the binding of ^{125}I -labelled IgG to chick yolk sac was reported to be 2.0×10^{-6} M (Tressler *et al.*, 1982), which is almost identical to the value obtained (at 4°C) in the present study for guinea pig yolk sac.

The uptake of IgG was investigated by exposing untreated yolk sac to ^{125}I -labelled ligand at 37°C. The amount of radioactivity specifically associated with tissue under these conditions exceeded the surface binding capacity, but was reduced if metabolic inhibitors were present in the medium. These results are consistent with the binding and active internalization of IgG. Previous experiments *in vitro* (King, 1977, 1982) have demonstrated the internalization of horseradish peroxidase-conjugated IgG by guinea pig yolk sac. Active internalization continued for several hours as indicated by the time-dependent uptake of ^{125}I -labelled IgG and the inert fluid-phase marker

[^{14}C]sucrose. Interestingly, cycloheximide had no effect on the uptake of ^{125}I -labelled IgG for at least 2 h, although protein synthesis was almost completely inhibited within 20 min. This suggests that binding and internalization are not primarily dependent on new receptor synthesis and implies that receptors are either recycled or that there is a pre-existing, intracellular pool from which the plasma membrane is replenished.

The finding that the endocytic index for ^{125}I -labelled IgG was greater than that of [^{14}C]sucrose is consistent with the involvement of an adsorptive component in the uptake of the former. On the assumption that the endocytic index for [^{14}C]sucrose defines fluid-phase pinocytosis, it can be seen that about 76% of the ^{125}I -labelled IgG uptake is adsorptive in nature. Since the competitive binding and uptake studies indicate that receptor-mediated uptake accounts for about 50% of total uptake, it is apparent that non-specific uptake, as measured here, must include both an adsorptive and a fluid-phase component. The endocytic index obtained for [^{14}C]sucrose in the present study is similar to that reported for sucrose uptake in the rat yolk sac and other systems (Pratten *et al.*, 1980). Weisbecker *et al.* (1983) reported values of between 9.1 and 13.4 $\mu\text{l/h}$ per mg of protein for the endocytic index of ^{125}I -labelled IgG incubated with rat yolk sac in the presence of calf serum, which are higher than the values obtained here for the guinea pig yolk sac. The difference may be attributable to the fact that a lower ligand concentration (1 $\mu\text{g/ml}$) was used in the rat yolk sac experiments.

Degradation and release studies

The inhibitory effect of leupeptin suggests that degradation of ^{125}I -labelled IgG is mediated, at least in part, by cysteine proteinases within the lysosomal system. The absence of any measurable effect of pepstatin could indicate either that cathepsin D activity is not rate-limiting for IgG breakdown or that the drug failed to reach the site of proteolysis. Unlike leupeptin, which diffuses rapidly into cells (Dunn & Aronson, 1977; Seglen *et al.*, 1979; Dean, 1979), pepstatin appears to enter cells by pinocytosis and may take several hours to attain effective concentrations within lysosomes (Dean, 1979). NH_4Cl and chloroquine were also effective inhibitors of ^{125}I -labelled IgG degradation. These compounds can diffuse into lysosomes, bind protons and so raise the intralysosomal pH (Ohkuma & Poole, 1978) with a resultant inhibition of hydrolytic activity. There is also evidence suggesting that certain amines may prevent the transfer of ligands between intracellular compartments, such as between endosomes and lysosomes (see Dean *et al.*, 1984). Either

or both explanations could account for the inhibition of degradation seen here. The role of the lysosomal system in IgG degradation suggested by these results is consistent with previous electron microscope studies from this laboratory (King, 1977, 1982). These studies showed that peroxidase-labelled IgG was internalized by yolk sac endodermal cells via a system of coated pits and vesicles and that some of these vesicles rapidly lost their coats and fused with lysosomes.

Our previous electron microscope studies also revealed peroxidase reaction product in coated vesicles which did not lose their coats and which seemed to fuse with the basolateral plasma membrane with release of reaction product into the lateral space. These results, and similar observations made in the rabbit yolk sac (Moxon *et al.*, 1976), were interpreted as showing coated vesicle-mediated transport of IgG. The experiments presented here show that yolk sac which had been pre-loaded with ^{125}I -labelled IgG released apparently intact ligand into the medium. Although it is tempting to ascribe this to transport, it is important in experiments of this kind to be able to assess the extent to which ligand has simply fallen off the tissue surface. Since the specific binding of IgG to paraformaldehyde-treated and to untreated yolk sac is essentially identical, it would be expected, if release was due only to loss of surface-bound ligand, that the amounts released from treated and untreated tissue would also be the same. This, however, was found not to be the case. Although much of the ^{125}I -labelled IgG released from untreated yolk sac had simply been lost from surface binding sites, at least 20% could not be accounted for in this way and can be considered as being due to exocytosis (transport) of ligand.

Evidence that receptor-mediated transport was contributing to the release of intact ligand is provided by the observations that, firstly, release was reduced if excess, unlabelled IgG was present during the period of exposure to ^{125}I -labelled IgG and, secondly, the amounts of ^{125}I -labelled IgG specifically released exceeded the specific surface binding capacity of the tissue. Furthermore, unlike specific binding, specific release showed no evidence of saturation and, in this respect, closely resembled the specific uptake pattern. While the results suggest that only a fraction of the total ^{125}I -labelled IgG taken up by the guinea pig yolk sac is transported and released in an intact form, it is difficult to give a precise figure without a more detailed analysis of rates of ligand dissociation and rates of ligand internalization. Intact IgG has also been shown to be released from the rat yolk sac *in vitro* (Williams & Ibbotson, 1979; Weisbecker *et al.*, 1983), although only total release was measured. One of the criteria used as evidence of

exocytosis was that release was inhibited at low temperature. Although a reduction in temperature would be expected to inhibit exocytosis of IgG, we found that it also reduced the rate at which ligand dissociated from both non-specific and specific surface binding sites. Thus, this criterion, at least, must be used with caution and, in the guinea pig, does not provide a valid means of distinguishing transport from the surface desorption phenomena. In the rat, 40% of the initial dose of IgG taken up was released in an intact form in 90 min, compared with a figure of 17% for the guinea pig. Apart from species differences, other likely reasons for the disparity are that much lower ligand concentrations and shorter washing times were used for the rat yolk sac experiments than were used in the present study.

One feature of the coated vesicle pathway suggested for IgG transport in guinea pig and rabbit yolk sac is that ligand destined to be transported is never exposed to lysosomal proteinases. An earlier model, proposed by Brambell (1966), implies that ligand destined for transport is exposed to proteinases but is protected by virtue of being receptor-bound. If the Brambell model is correct, inhibitors of proteolysis would spare unbound IgG from degradation and so allow more protein to be transported. However, under conditions in which the degradation of ^{125}I -labelled IgG was inhibited by between 40 and 67%, no concomitant increase in the amount of intact ligand release was detected (Table 2), suggesting that transport is independent of proteolysis and pointing in favour of the coated vesicle hypothesis. Essentially identical results were reported for the rat yolk sac (Weisbecker *et al.*, 1983) although the nature of the transporting vesicle remains undefined in this system.

In contrast to homologous IgG, a different pattern of processing was found for bovine IgG and bovine serum albumin. The relative degradation of both these proteins was greater than homologous IgG and no evidence of transport was found, although relatively large amounts appeared to be lost to the incubation medium by surface desorption. This pattern is not inconsistent with the limited data from experiments *in vivo* which suggest that, at best, only small amounts of bovine IgG and bovine serum albumin reach the fetal circulation after injection into pregnant guinea pigs (Al-Nadji, 1965; Kulangara & Schechtman, 1963).

This work was supported by NIH grant ND 11658.

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