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Modulation of systemic cytokine levels by implantation of alginate encapsulated cells

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Abstract

The availability of cell lines that are transfected with IL-4, IL-5 and IFN-γ cytokine genes permits the prolonged in vivo delivery of functional cytokines in relatively large doses for the modulation of specific immune responses. Often the transfected cells are xenogeneic or allogeneic to the experimental animal and have to be encapsulated in such a way that no cellular response by the host will be induced. Alginate has proven to be a simple matrix for encapsulating cells under mild conditions suitable for in vivo implantation. Encapsulated cells express the transfected IL-4 gene for at least 14 days after in vivo implantation and were shown to be functional during that period by modulating ongoing IgE responses. The application of adherent growing transfected cells permits dose-response titrations and provides an easy method for local and systemic cytokine delivery. Alternatively, hybridoma cells can be encapsulated and the secreted antibody monitored in the serum. It was found that no host immune response was triggered by alginate encapsulated cells. The efficiency of treatment by encapsulated hybridoma cells was shown to be equivalent to that of injecting purified antibodies.

Key words: Alginate encapsulation; Cytokine transfected cell line; Hybridoma

1. Introduction

The immunoregulatory role of cytokines in immune responses is well established. A special interest has developed in the modulation of immune responses in experimental animal model systems by treatment with cytokine-neutralizing monoclonal antibodies (mAb) or recombinant cytokines in vivo. This can be achieved by repeated injections of relatively large amounts (mg) of purified mAb. Alternatively, multiple injections with several μg of purified recombinant cytokines have to be given each day to induce a detectable effect. Major drawbacks to the application of these techniques are the large amounts of highly purified mAb or recombinant cytokines required, the need to use large scale purification procedures (with problems of yield, recovery, and endotoxin contamination of biologically active mate-

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Abbreviations: IL, interleukin; IFN-γ, interferon-γ; mAb, monoclonal antibody.

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rial) and the short biological half life of most cytokines (minutes) in the circulation that necessitate multiple injections each day.

IL-4 is obligatory for the induction of IgE synthesis as evidenced by its ability to induce the expression of ϵ germline transcripts (Rothman et al., 1988; Yoshida et al., 1990), the inhibition of parasite-induced IgE responses by neutralizing antibodies against IL-4 (Finkelman et al., 1990), the absence of IgE responses in nematode-infected mice made IL-4 deficient by gene targeting (Kuhn et al., 1991) and the hyperproduction of IgE in IL-4 transgenic mice (Tepper et al., 1990; Muller et al., 1991).

The availability of cell lines that are transfected with cytokine genes enables the in vivo application of functional cytokines in doses sufficient to modulate specific immune responses (Lee et al., 1986; Yokota et al., 1987; Karasuyama and Melchers, 1988). The implantation of these cell lines in vivo can be used as an alternative to cytokine transgenic animals without the potential drawbacks of developmental disturbances (Tepper et al., 1990; Muller et al., 1991). However, the transfected cells will often be xenogeneic or allogeneic to the experimental animal and have to be encapsulated in such a way that no cellular response by the host will be induced. Encapsulation in alginate provides a simple, suitable method for immobilizing viable cells under mild conditions for in vivo implantation (Kupchik et al., 1983; Kierstan and Coughlan, 1985; Bucke, 1987).

This study describes the combination of adherent growing cytokine gene transfected cell lines and alginate encapsulation for the in vivo modulation of the murine IgE response.

2. Materials and methods

2.1. Mice

Female BALB/c, SJA/9 and SJL mice were bred and kept in our own colony and used at an age of 12–16 weeks. Animals were kept in light-cycled rooms and had free access to acidified water and food. The microbial status of the mice fulfilled the standard of 'specific pathogen free V' according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments.

2.2. Antibodies

Rat monoclonal IgG1 antibodies specific for murine IL-4 (11B11, Ohara and Paul, 1987), IL-5 (TRFK 5, Schumacher et al., 1988) IFN-γ (XMG 1.2, Cherwinski et al., 1987) and *E. coli* β-galactosidase (GL 113, used as an isotype control, Chatelain et al., 1992) were obtained by purification from the supernatants of stable hybridoma subclones grown in defined RPMI 1640 medium supplemented with pyruvate (0.1 M), L-glutamine (4 mM), antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin) and 1% (v/v) Nutridoma SP (Boehringer, Mannheim, Germany). The supernatants were concentrated approximately 20 times by 10000 Da ultrafiltration (Amicon, Danvers, MA). The mAb were purified by saturated (20% w/v) sodium sulphate precipitation and dialysis into 50 mM sodium acetate buffer, pH 4.9. After centrifugation at 10000 rpm to remove precipitated albumin the antibodies were further purified by affinity chromatography on a 10 ml bed volume of Immunopure protein G (Pierce, Rockford, IL) using the same acetate buffer as loading buffer and 0.1 M glycine-HCl, pH 2.5 as elution buffer. After neutralization with 1 M Tris, the sample was dialysed into phosphate buffered saline, concentrated by ultracentrifugation and filter sterilized through a 0.22 μm Millex GV filter (Millipore, Bedford, MA).

2.3. Cell lines

The above mentioned hybridoma cell lines were kind gifts of Dr. R.L. Coffman (DNAX Research Institute, Palo Alto, CA). Cultures of these hybridoma cell lines were also used for alginate encapsulation. Stable transformants of the monkey CV1 cell line were obtained by transfecting the murine IL-4 or IL-5 gene and placing the gene under control of the SV40 promotor. Alternatively CHO-Ki cells were stably transfected with the murine IFN-γ gene. All the transfected cell lines were kind gifts of Dr. N. Arai

(DNAX). These adherent cell lines were grown on Cytodex 3 beads (Pharmacia, Uppsala, Sweden) in Dulbecco's modified medium (DMEM) containing 10% FCS, glutamine and antibiotics. Cells were kept in exponentional growth phase by splitting the cultures every 3 days. The TNP-specific mouse IgE secreting IgELA2 cell line was a kind gift of Dr. M. Wabl (Rudolph et al., 1981).

2.4. Alginate encapsulation

Cultured transfected cells grown on Cytodex beads were washed extensively with cold sterile saline and mixed with two parts of a sterile 1.2% alginate solution (FMC, Vallensbaek Strand, Denmark) that was warmed to room temperature. The mixture was transferred into a syringe of suitable size equipped with a 23–27 gauge needle depending on the application (refer results section). Next, the mixture was injected into a fresh solution of 80 mM $CaCl_2$ (prepared in water) at room temperature with continuous gentle mixing. The capsules were washed with cold saline and a 1 ml volume containing 2×10^6 cells (or other cell concentrations as indicated in the results section) was injected intraperitoneally (i.p.) or subcutaneously (s.c.) as indicated. Alternatively, cultured hybridoma cells could also be encapsulated using 27 gauge needles. The capsules could be recovered for periods up to 18 days after injection by peritoneal washing. The cells could be isolated by incubating the capsules for 5 min at 37°C with a 1% trypsin-EDTA mixture (Gibco, Grand Island, NY), thereby establishing that these capsules remained intact in the mouse and that the encapsulated cells were viable for this period in vivo and were still able to produce cytokines in vitro after their recovery.

2.5. Assays

Mice were immunized with 10 μg TNP-KLH adsorbed on alum i.p.. Total serum IgE levels were measured by an isotype-specific ELISA as described previously (Coffman and Carty, 1986). TNP-specific IgE was quantitated in a modified sandwich ELISA. Plates were coated with the same rat monoclonal anti-mouse IgE antibodies (EM95, 2 μg/ml) and incubated overnight at 4°C with diluted serum samples. TNP modified with alkaline phosphatase (kind gift of Dr. A.J.M. van den Eertwegh, MBL-TNO, Rijswijk, Netherlands) was then added. The ELISA was further developed using Sigma 104 phosphatase substrate (Sigma). The detection limit of this ELISA was 0.2 ng/ml. IL-4, IL-5 and IFN-γ were measured by a sandwich ELISA as described previously (Schumacher et al., 1988; Cherwinski et al., 1987; Chatelain et al., 1992). Hybridoma protein produced by encapsulated hybridoma cells was measured in a rat IgG1-specific ELISA by coating plates with 4 μg/ml of a purified rat IgG1-specific mouse monoclonal antibody Mr G1P (clone MRG 1; Pharmingen, San Diego, CA) and incubating with appropriate dilutions of serum. Subsequently, 0.5 μg/ml of a biotin-conjugated mouse anti-rat IgL (κ chain) monoclonal antibody (clone MRK 1; Pharmingen), a 1/1000 dilution of a streptavidin horseradish peroxidase conjugate (Jackson Immunological Laboratory, Westgrove, PA) and the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma, St. Louis, MO) were added. A purified rat IgG1 (clone R3 34; Pharmingen) was used as an isotype-specific standard. The detection limit of this ELISA was 1.25 ng/ml of rat IgG1 in mouse serum and there was no cross-reactivity with murine IgG1. Substituting the coat with an *E. coli* β-galactosidase-specific rat IgG1 mAb (GL113) revealed no signal due to idiotypic activity. In order to detect rat IgG1-specific immune responses in the treated mice, serum dilutions were incubated on ELISA plates coated with 4 μg/ml of rat IgG1 (11B11). For the detection of peroxidase-labelled goat anti-mouse IgM or IgG antibodies (Southern Biotechnology Associates, Birmingham, AL) were used at a 1/1000 dilution. The derived serum concentrations were expressed as arithmetic means (± 1 SD).

2.6. Reverse type PCR analysis of IL-4 gene expression

Cells were collected and resuspended in 0.5 ml of 4 M guanidinium thiocyanate (GTC; Fluka, Buchs, Switzerland) and either analysed directly

or frozen at −70°C until further analysis. Total cellular RNA was isolated after making up the volume of the sample to 2.5 ml with GTC according to the method of Chirgwin et al. (1979). Gradient centrifugation was performed on an equal volume of 5.7 M cesium chloride for 18 h in an ultracentrifuge (Sorvall-Du Pont, Newtown, CT). After ethanol precipitation, 1 μg RNA was used in a reverse transcriptase reaction, modified from the procedure of Krug and Berger (1987). Briefly, 0.01 U oligo(dT)$_{15}$ (Pharmacia, Uppsala, Sweden) was added to a final volume of 14 μl and both RNA and oligo(dT)$_{15}$ were heated for 3 min at 85°C. Then the oligo-primed RNA was added to a mixture containing 1 × avian myoblastoma virus reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EDTA (ethylenediamine tetraacetate disodium dihydrate salt); 1 μg/ml bovine serum albumin), 1 mM deoxy nucleotide triphosphate (dNTP), 4 mM sodium pyrophosphate, 40 U RNAsin (Promega, Madison, WI) and 5 U avian myoblastoma virus reverse transcriptase (Boehringer Mannheim, Germany). This mixture was incubated for 1 h at 39°C. From this cDNA mixture 5% was used in a PCR reaction. To this end, the cDNA was mixed with 1 × Taq buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin), 0.2 mM dNTP, sense and anti-sense primers ($OD_{0.2}$) and 1 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). For amplification, 35 cycles (1 min at 94°C for denaturation, 2 min at 55°C for annealing and 3 min at 72°C for primer extension) were performed, using a Perkin-Elmer Cetus DNA thermal cycler. For all samples, an IL-4 sense and anti-sense primer set and a sense and anti-sense primer set for the HPRT (hypoxanthine phosphatidyl ribosyltransferase) housekeeping gene were used.

2.7. Staining

Cell suspensions were stained with anti-I-A^d (clone 39-10-8, Pharmingen, San Diego, CA) mAb as described previously (Knulst et al., 1991). Cells were analysed using a flow cytofluorometer (FACScan, Becton Dickinson, Mountain View, CA).

3. Results

3.1. Cytokine transfected cells or hybridoma cells can be encapsulated

Alginate permits the encapsulation of both non-adherent hybridoma cells and adherent cytokine gene transfected cell lines. The latter cells can be grown on Cytodex beads and subsequently encapsulated in alginate as shown in Fig. 1. The difference in the cell source determines the minimum size of the needle employed in the encapsulation procedure, namely 25 or 27 gauge for hy-

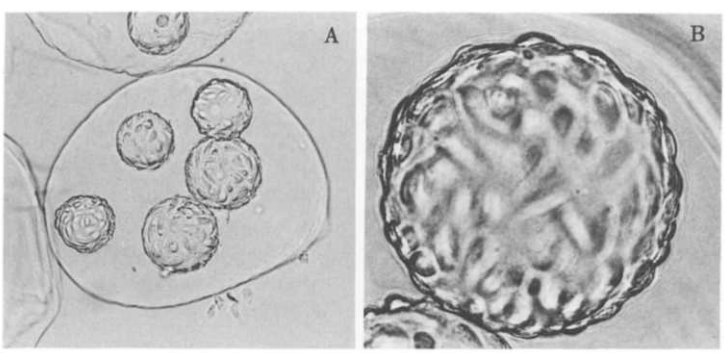

Fig. 1. *A*: photograph showing beads covered with IL-4 transfected CV1 cells encapsulated in alginate (40 × magnification). *B*: detail of a Cytodex bead fully covered with CV1/IL-4 cells (100 × magnification).

bridoma cells or 23 gauge for cells grown on beads (data not shown). The use of beads allows the exact dosage of cells implanted in vivo after encapsulation to be determined, since cells adhere to the beads only. Fully occupied beads grown with transfected CV1/IL-4 cells contained on the average 500 live cells per bead as determined by Trypan Blue exclusion after stripping the cells by incubation for 5 min in trypsin EDTA. Using a 23 gauge needle, capsules comprising, on average, five beads per capsule were obtained. This necessitates implantation of roughly 4000 beads per mouse in order to administer 2×10^6 cells.

To allow a sufficient degree of gelatination and hardening to occur, the capsules were incubated for some time in a $CaCl_2$ solution. This treatment was not detrimental to the viability of the encapsulated cells. In order to determine the optimal incubation time, XMG 1.2 cells were encapsulated and the capsules were incubated for various periods in the $CaCl_2$ solution after which the capsules were dissolved with trypsin-EDTA. The viability of the cells was determined by trypan blue exclusion. The results (Table 1), showed that viability during incubation times of 1 min up to 15 min did not differ significantly. In subsequent experiments the capsules were incubated for 10 min.

Table 1
Effect on cell viability of hardening of alginate capsules in $CaCl_2$

Time (min)	Viability (%)
0	82
1	60
2	62
5	50
10	44
15	50

XMG1.2 cells encapsulated in alginate were maintained in fresh 80 mM $CaCl_2$ solution prepared in water. Viability is expressed relative to the starting cell population.

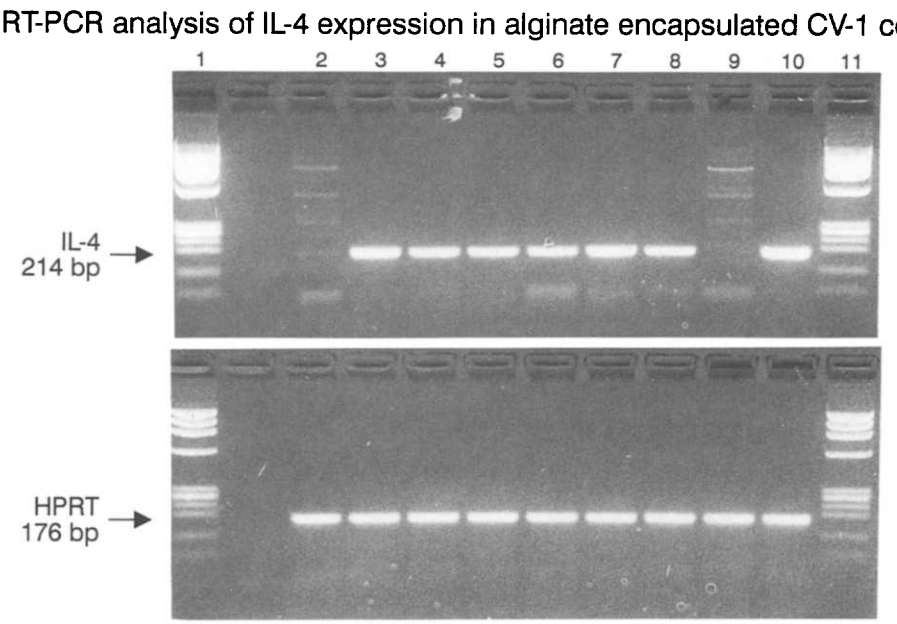

Fig. 2. Alginate encapsulated CV1/IL-4 cells (2×10^6) were implanted (i.p.). Capsules were washed out of the peritoneum on the days indicated and cells were detached from the beads. Total RNA was extracted, cDNA was prepared and reverse type PCR analysis was performed using an IL-4 primer set (*A*) or HPRT primer set (*B*). Lane 1: base pair marker (Phi X174 RF DNA HaeIII digest); lane 2: mock transfected control; lanes 3–8: CV1/IL-4 cells washed out on days 0, 3, 5, 7, 10 or 14; lane 9: mock transfected control; lane 10: positive control (CDC 35 T cell clone); lane 11: bp marker.

3.2. Encapsulated cells secrete cytokines or antibodies in vivo

The in vivo production of cytokines by encapsulated cytokine-transfected cells cannot be measured in the serum due to the abundant presence of inhibitors, such as soluble cytokine receptors. Alternatively, despite the fact that cytokine is continuously released in vivo at a single time point, not enough IL-4 may be present in the serum to be detected in our assay. This could be the result of a low secretion rate, analogous to IL-6 (Shirai et al., 1993). Therefore, we analysed the mRNA expression of IL-4 by encapsulated CV1/IL-4 cells that were washed out of the peritoneal cavity on various days after implantation. The results (Fig. 2) show that even up to 14 days after implantation IL-4 mRNA could still be detected. After day 14 the level of expression of both IL-4 mRNA and HPRT mRNA slowly declined suggesting increasing cell death.

In order to determine whether encapsulated hybridoma cells were still able to secrete their mAb, 2×10^6 TNP-specific mouse IgE secreting IgELA2 hybridoma cells were injected into BALB/c mice. Serum analysis of total and TNP-specific IgE showed up to a 10-fold increase in total IgE level after 6 days due to a 100-fold increase in TNP-specific IgE antibodies (Table 2). The serum IgE level remained elevated for up to 32 days, although gradually declining after 14 days.

The injected hybridoma cells carried the H-2^d haplotype that is compatible with the haplotype

Table 2
TNP-specific IgE levels in the serum of IgELA2 hybridoma-bearing mice

Day	IgE (μg/ml)		Ascites incidence
	Total	TNP-specific	
0	1.11 ± 0.42	< 0.005	0/10
6	4.29 ± 1.13	1.44 ± 0.37	0/10
14	8.45 ± 1.69	5.36 ± 1.21	2/10
22	8.63 ± 1.24	6.54 ± 1.15	6/10
32	2.86 ± 0.87	1.04 ± 0.17	10/10

BALB/c mice were implanted i.p. with 2×10^6 TNP-specific IgELA2 cells encapsulated in alginate. Serum IgE levels, expressed in μg/ml, are arithmetic means ± 1 SD ($n = 10$).

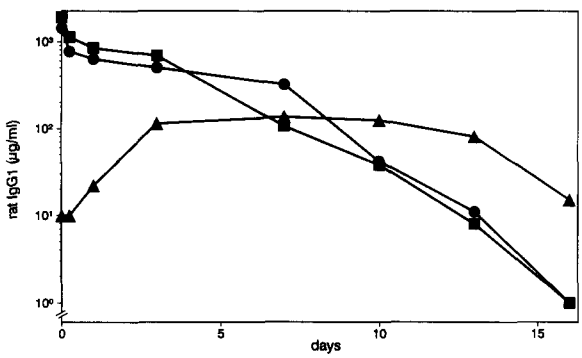

Fig. 3. Kinetics of clearance of rat IgG1 from mouse serum. BALB/c mice were injected with either 1.5 (squares) or 2 mg (circles) purified XMG 1.2 mAb or implanted i.p. with 2×10^6 encapsulated cells (triangles). Serum levels of rat IgG1 were monitored by specific ELISA. Results are expressed as means ($n = 5$).

of the BALB/c mice used. Hence there was rapid development of peritoneal tumors accompanied by the formation of ascites fluid. This suggests that upon leakage or breaking of the alginate capsules, the hybridoma cells are able to induce ascites formation. However, as shown in Table 2, ascites formation occurred, relatively late (more than 14 days) after implantation of encapsulated hybridoma cells.

Repeating the experiment in SJL mice, peritoneal cells were harvested on days 3, 5 and 7 after implantation and the non-encapsulated cells were stained for the H-2^d haplotype. Subsequent FACScan analysis failed to reveal significant amounts (> 5%) of free hybridoma cells (data not shown). We therefore assume that leakage of actively secreting cells from the capsule is not significant.

3.3. Kinetics of the in vivo production by encapsulated cells

To determine the rate at which rat IgG1 appeared in the serum after implantation of encapsulated hybridoma cells, kinetic experiments were performed. As shown in Fig. 3, significant levels of rat IgG1 were detected in the serum after 3 days, peaking around day 8, and lasting for at least 13 days. For comparison, mice were injected

once with either 1.5 or 2 mg of purified XMG 1.2 (rat IgG1) antibodies (Fig. 3). In these cases a steadily declining serum level of rat IgG1 was observed. From the data obtained the rate of disappearance ('biological half life') of rat hybridoma IgG1 in mouse serum in vivo was estimated to be of the order of 4.5 days. This figure was calculated from the data obtained after injection of purified hybridoma protein as well as from the data obtained after implantation of encapsulated hybridoma cells. In addition, in the case of rat IgG1 production by encapsulated hybridoma cells in vivo, the synthetic rate as determined by the increase in serum level was estimated to be of the order of 1.5 days. As is evident from Fig. 3, implantation of 2×10^6 encapsulated hybridoma cells provided a serum level of rat IgG1 that was lower than that obtained by injecting 1.5 mg purified IgG1. On the other hand the serum levels of rat IgG1 were much more prolonged after implantation than after injection of purified rat IgG1. Collectively, these results suggest that implantation of alginate encapsulated cytokine transfected cells or hybridoma cells provides an efficient method for cytokine or antibody treatment in vivo for periods of up to 14 days.

3.4. Dose-response titration of encapsulated cells

In order to determine the amount of hybridoma protein present in the serum of mice implanted with various doses of encapsulated cells, dose titrations were performed. To this end, encapsulated rat IgG1-secreting hybridoma cells specific for the cytokine IL-5 were implanted. The appearance of rat IgG1 in the serum of these mice was determined by ELISA and the persistence of this rat Ig was studied. As shown in Fig. 4*A*, the injection of 2×10^6 encapsulated cells resulted in the appearance in the serum of hybridoma protein at concentrations up to 100 μg/ml. These levels were reached after 7 days and lasted for up to 21 days, after which they steadily declined. A dose of 3×10^3 cells resulted in a 1000-fold lower serum level of hybridoma protein that did not show clear kinetics and was at the limit of detection of the ELISA. The other doses tested resulted in intermediate serum levels of rat IgG1. The maximum increase in rat IgG1 levels in the serum occurred between days 7 and 21, suggesting that the implanted cells were still actively secreting the hybridoma protein (Fig. 4*B*).

3.5. Implantation sites of encapsulated cells

All of the results described above were obtained by i.p. implantation of encapsulated cells. This site can easily accommodate 1 ml samples. Moreover, multiple implantations of 1 ml samples can be performed i.p. when using 2 week intervals. For applications in which a single implantation is to be used the s.c. site may be preferable.

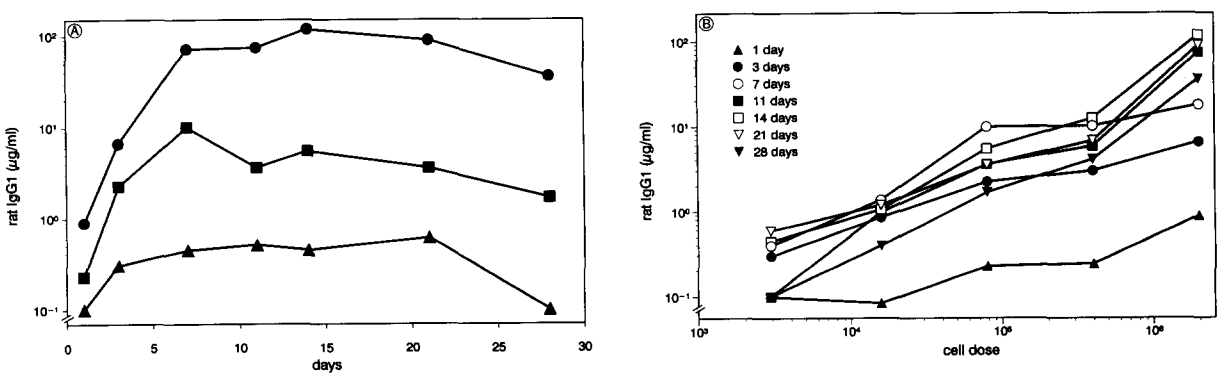

Fig. 4. Kinetics of cell dose-dependent rat IgG1 production. BALB/c mice were implanted i.p. with encapsulated TRFK 5 cells and rat IgG1 production was monitored by ELISA. *A*: serum levels of hybridoma protein at various intervals after implantation of alginate encapsulated cells. Doses of 2×10^6 (circles), 8×10^4 (squares) and 3×10^3 (triangels) encapsulated TRFK5 cells were used. *B*: serum levels of hybridoma protein at various doses of the alginate encapsulated cells. Serum levels were determined 1, 3, 7, 11, 14, 21 and 28 days after implantation as indicated. Results are expressed as mean serum levels ($n = 5$).

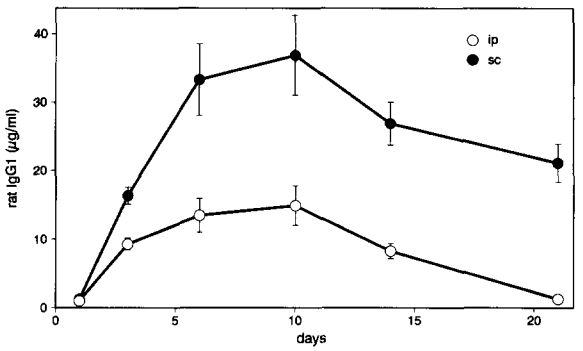

Fig. 5. Effect of implantation site on the production of hybridoma protein. BALB/c mice were implanted i.p. (open symbols) or s.c. (closed symbols) with 2×10^6 encapsulated 11B11 cells. Rat IgG1 levels were monitored by a specific ELISA. Results are expressed as mean serum levels (± 1 SD) ($n = 10$).

To analyze efficacy in terms of long term antibody production, both sites of implantation were compared. To this end, 2×10^6 11B11 cells were encapsulated and implanted either i.p. or s.c.. Again, rat IgG1 levels were monitored. Fig. 5 shows that s.c. implantation resulted in significantly higher and more sustained antibody production than i.p. implantation. The s.c. site was therefore considered to be superior for single implantation and cells were found to remain active for periods up to 1 month (data not shown). The i.p. site, on the other hand, was found to be more suitable for multiple injections (every 14 days) which permits cytokine or antibody treatments over prolonged periods of time.

3.6. Mouse anti-rat response after implantation of encapsulated cells

Since mice can be treated for long periods of time with encapsulated hybridoma cells that provide low but persistent levels of hybridoma protein, a host anti-rat response could be expected to occur. In order to investigate the occurrence of such a response, BALB/c mice were immunized with 10 mg TNP-KLH on alum i.p. 3 months later mice were boosted with a similar dose of antigen. During the whole immunization period the mice were treated every other week with 2×10^6 encapsulated 11B11 cells implanted i.p. In total the mice received seven repeated injections. As is evident from Fig. 6, only a minor rat IgG1-specific IgM response developed during the booster response. No IgG anti-rat IgG1 response could be detected in these mice (data not shown). Monitoring of the serum revealed around 10 μg/ml of rat IgG1 was present in the mouse serum (data not shown).

Table 3
Cytokine-dependent modulation of primary IgE responses by encapsulated cells

Strain	TNP-KLH immunization	Encapsulated cells	Antibody treatment	Anti-TNP IgE (ng/ml)	
				Day 0	Day 14
BALB/c	–	–	–	15	18
	+	–	–	20	1947
	+	CHO/IFN-γ	–	18	21
	+	CHO/IFN-γ	XMG1.2	17	2354
	+	11B11	–	32	28
	+	–	11B11	25	27
	+	Capsules only	–	29	32
SJA/9	+	–	–	32	25
	+	CV-1/IL-4	–	19	2048
	+	CV-1/IL-4	GL113	19	2123
	+	CV-1/IL-4	11B11	21	20
	+	CV-1/IL-5	–	16	20
	+	GL113	–	20	29

Mice were immunized (i.p.) with 10 μg TNP-KLH in alum on day 0. Mice were treated with 2×10^6 alginate encapsulated cells implanted i.p. and/or with 2 mg of purified XMG1.2 or GL113 or 10 mg of 11B11 mAb i.p. Serum levels were determined by TNP-specific IgE ELISA. Results are reported as arithmetic means ($n = 5$).

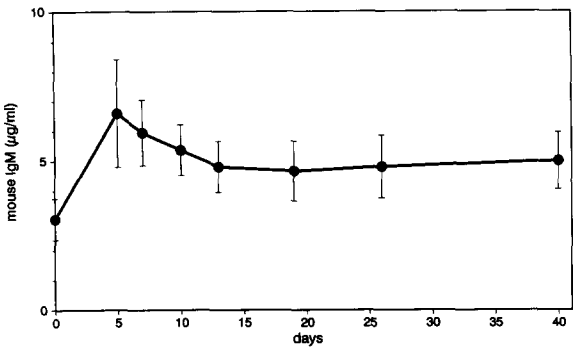

Fig. 6. Mouse anti-rat response after implantation of rat IgG1-secreting hybridoma cells. BALB/c mice were implanted with 2×10^6 encapsulated 11B11 cells i.p. Mouse IgM anti-rat IgG1 responses were determined in an ELISA procedure. Results are expressed as means $\pm$ 1 SD ($n = 5$).

3.7. Modulation of IgE formation in vivo by encapsulated cells

In order to test the possibilities of modulating in vivo immune responses, e.g., IgE formation, mice were immunized and implanted with encapsulated fibroblast cells transfected with cytokine genes or hybridoma cells. IgE high responder BALB/c or non-responder SJA/9 mice were immunized with 10 μg TNP-KLH on alum i.p. These mice were implanted i.p. with encapsulated CV1 cells transfected with the IL-4 or the IL-5 gene or with CHO cells transfected with the IFN-γ gene. Subsequently, these mice were treated with 10 or 2 mg of purified anti-IL-4 or anti IFN-γ mAb i.p. respectively, doses widely shown to be sufficient to block IL-4 or IFN-γ-mediated effects in vivo. As an isotype control, mice were treated with GL113 mAb. The resulting TNP-specific IgE levels in the serum were analysed by ELISA. As shown in Table 3, the resulting TNP-specific IgE response in BALB/c mice could be blocked by IFN-γ secreting cells, anti-IL-4 secreting cells or purified anti-IL-4 mAb. Blocking the activity of IFN-γ by treatment with purified anti-IFN-γ mAb resulted in a marked increase in TNP-specific IgE to levels higher than those obtained by TNP-KLH immunization alone. SJA/9 mice could overcome the defective IgE production following implantation with IL-4-transfected cells, and the subsequent IgE production could be blocked by anti-IL-4, suggesting an IL-4-mediated IgE production. The results in Table 3 also show that implanting 2×10^6 11B11 or XMG 1.2 hybridoma cells resulted in in vivo effects that were at least comparable to those obtained by injecting 2 mg of XMG 1.2 or even 10 mg of 11B11 per mouse. These doses have been widely shown to be sufficient to block cytokine mediated effects in vivo. Moreover, these results show that injection of encapsulated empty Cytodex beads or IL-5 transfected CV1 cells did not lead to IgE production in SJA/9 mice.

4. Discussion

This study has shown that alginate encapsulation of cytokine transfected cells offers an easy way to establish a constant systemic level of cytokine in vivo for prolonged periods of time. Moreover, the use of adherent growing transfected fibroblasts in combination with Cytodex beads provides an exact dosage of implanted cells and allows precise dose-response relationships to be defined. Although hybridoma cells can also be encapsulated effectively in alginate, growth will continue until limited by physical parameters, such as charge repulsion between the net negative surface of cells and the negatively charged carbohydrate groups of the alginate (Haug and Smidsrod, 1967). The leakage of entrapped cells out of the capsules is undetectable, at least to day 7. Therefore, cells released from the capsules resulting from breaking or dissolving the capsule will not remain viable and thus such cells will not contribute to the observed serum levels of hybridoma protein. The maximum numbers of cells that can be encapsulated is largely determined by the low gel strength of the capsules and by certain diffusion restrictions; mainly for oxygen (Cheetham et al., 1979). Determination of an exact dosage of implanted hybridoma cells is virtually impossible, since the implanted capsules permit further proliferation until limited by physical constraints. On the other hand, the activity of implanted hybridoma cells can be measured easily by monitoring the serum for the presence of

the secreted antibodies. As shown here, the rate of disappearance of rat IgG1 delivered in this way is roughly 4.5 days, which is an estimate of the biological half-life and equivalent to the half life estimated by the injection of purified rat mAb (Wawrzyneczak et al., 1992). Recently, an alternative method was described to deliver some cytokines for periods up to 9 days by complexing low doses of cytokine with either neutralizing antibodies or soluble receptors (Sato et al., 1993). A potential problem arising from the implantation of hybridoma cells for in vivo antibody production, certainly for anti-cytokine antibodies, is cytokine production by the hybridoma cells themselves. As has been described, hybridoma cells can secrete relatively large amounts of IL-6 and although in this study rat hybridomas were used in mice, there can be sufficient cross-reactivity to induce interfering unknown bystander effects (Van Damme et al., 1987).

As shown here, the implanted capsules stay intact and the cells continue to secrete their cytokine for up to 3 weeks. Furthermore, using an ELISA specific for soluble mouse-anti-rat IgG1 antibodies does not reveal a detectable anti-rat response in the host triggered by encapsulated cells. The ELISA employed, however, would not be able to detect anti-rat antibodies complexed to the rat hybridoma protein. Such complexes could consist of anti-isotypic antibodies or anti-idiotypic antibodies specific for rat IgG1. The use of an irrelevant rat IgG1 for coating did not reveal any signal in the serum of treated mice, suggesting that either large complexes are formed (due to anti-isotypic antibodies) in the serum or substantial amounts of anti-idiotypic antibodies are present that cannot be detected. The serum levels of rat IgG delivered by alginate encapsulated hybridomas were relatively low (mostly ≤ 25 μg/ml), and this IgG was only induced in the absence of an adjuvant. Although the serum level of rat Ig was sufficient to block the specific ELISA, we consider the induction of an anti-rat Ig response unlikely within the limitations of our detection technique. Moreover, the decrease in serum levels of rat IgG1 after 14 days was reflected in the parallel deterioration of the alginate capsules and the increased death of hybridoma cells. This makes it unlikely that a mouse anti-rat response (both idiotypic and isotypic) would be responsible for the decrease in rat IgG serum levels.

Complexes of cytokines with anti-cytokines have been shown to increase the half life of cytokines in the serum (Sato et al., 1993). Anti-cytokine antibodies (delivered by alginate capsules) should be active in their depleting capacity for periods of more than 2 weeks but this has not been observed. Moreover, the injection of rat purified IgG1 antibodies did not induce an anti-rat immune response despite the initial high doses of antibody present. Since no adjuvant was used, the serum half life of 4.5 days ensured a rapid decline to levels comparable to those in alginate implanted mice. In another study (Van Ommen et al., submitted) mice were treated at bi-weekly intervals with 10 mg purified 11B11 per mouse for a period of 3 months. In these mice no significant rat IgG1-specific IgG response could be detected during this treatment and the rat IgG1 serum levels never exceeded 25 μg/ml. This makes it unlikely that an induced immune response would be missed using the ELISA. When other anti-mouse cytokine rat IgG mAbs were used, anti-rat IgG1 responses were not detected. This was probably due to the fast clearance of both uncomplexed and cytokine-complexed antibodies.

A medium for the entrapment of living cells should cause as little trauma to the cells as possible. There should, ideally, be no shock to the cells from change of temperature, chemical environment and osmotic pressure. Entrapment in calcium alginate meets all these criteria. Alginate, extracted with sodium hydroxide from *Macrocystis pyrifera*, consists of a copolymer of β-D-mannopyranosyl uronate and α-L-gulopyranosyl uronate linked by $(1 \rightarrow 4)$-glycosidic linkages. When exposed to divalent ions such as Ca^{2+}, an inert three-dimensional polymer gel network is formed with relatively large interconnected interstitial spaces (Bucke, 1987). The average diameter of the capsules (approximately 0.5–5 mm), is limited by the size of the needle and the pressure applied to the syringe. The main disadvantages of alginate capsules are (1) they are rapidly disrupted by chemicals capable of chelat-

ing calcium ions, and (2) the potential limitation of oxygen transfer into the capsule (Familetti and Fredericks, 1988; Hashimoto and Shirai, 1990). When comparing various implanted hybridoma cells, it appears that at maximum production these cells yield systemic levels of 10–100 μg/ml of rat IgG. This points to a limited production potential, possibly due to a lack of oxygen or reduced diffusion of nutrients and waste products.

IgE responses are dependent on the presence and activity of functional IL-4 as the most prominent IgE switch inducing factor. IFN-γ, being a major antagonist of IL-4, plays an important role in the downregulation of the IgE production (Finkelman et al., 1988; Mosmann and Coffman, 1989a). It has been suggested that the ratio of IL-4 to IFN-γ determines the ability to produce IgE in vivo (Snapper and Paul, 1987; Mosmann and Coffman, 1989b). Thus, implantation in IgE high-responder BALB/c mice of either cytokine transfected cells or hybridoma cells provides an effective model system to modulate the IgE response based on influencing the balance between essential cytokines in vivo. As is evident from the results (Table 3), the IgE response induced by immunization with TNP-KLH can effectively be inhibited by implantation of IFN-γ producing CHO/IFN-γ cells. Subsequent neutralization with purified anti-IFN-γ mAb (XMG 1.2) induced complete reversal of this inhibition. Implantation of anti-IL-4 secreting hybridoma cells (11B11) or injection of purified anti-IL-4 mAb resulted in a similar degree of inhibition to that observed with implanted CHO/IFN-γ cells. These findings are in complete agreement with the hypothesis that the relative amounts of functional IL-4 and IFN-γ are sufficient to determine the IgE response in vitro. IgE non-responder SJA/9 mice, on the other hand, do not react to TNP-KLH immunization with the formation of TNP-specific IgE. Following infection with the helminth parasite *Nippostrongylus brasiliensis* it has previously been shown that implantation of alginate encapsulated CV-1/IL-4 transfected cells was able to restore IgE formation to levels comparable to those in BALB/c mice (Savelkoul et al., 1991). Here, we were able to show that a similar treatment restored the formation of TNP-specific IgE. This SJA/9 model clearly shows that implantation of CV-1/IL-4 cells yields sufficient functional IL-4 to induce the formation of IgE. Collectively, these results illustrate the potential applicability of implantation of alginate encapsulated cytokine transfected cells in the modulation of immune responses in vivo.

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