In vitro cloning and regulatory potential of B cell hybridomas secreting entero-antigen specific antibody.

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Abstract

Enteroantigen specific B cells have previously been demonstrated to play an immunomodulatory role in experimental colitis such as the T cell transfer model of colitis and regulatory B cells are known to be important for suppressing immunopathology in human IBD. Establishing immortalized B cell hybridomas with enteroantigen specificity would be an important tool to investigate further the role of B cells in the immunopathology of experimental colitis and inflammatory bowel disease. Here, based on polyethylene glycol fusion of X63 cells and enteroantigen reactive murine $CD19^+$ B cells, we generated enteroantigen specific B cell hybridomas with proliferative capacity and IL-10 secretion against enteroantigen. The establishment of such hybridomas might be the basis for further assessing the enteroantigen specificity of gut-derived B cells, enteroantigens relevant for experimental colitis as well as human IBD including the immunoregulatory role of such enteroantigen specific B cells.

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Introduction

Apart from its immunoglobulin (Ig) secreting function, subsets of B cells, the so-called regulatory B cells (Bregs), also possess immune suppressive functions in mouse and man (1-5). Bregs have been shown to play a suppressive role in mouse autoimmune models such as experimental allergic encephalitis (EAE) (6,7), experimental arthritis (8,9), autoimmune diabetes (10) and in models of colitis (11,12) . In humans, Bregs appear to play a role in inflammatory bowel disease (IBD) because B cell depletion in patients with IBD tend to aggrevate the disease (13,14). The main mechanism of suppression is the secretion of anti-inflammatory molecules such as Interleukin (IL)-10 (4,5,15) and transforming growth factor (TGF)-β (1,16). In addition, in certain systems Bregs have been shown to cause T cell death by their expression of FAS ligand (17) and programmed death-ligand 1 (PD-L1) (18). Recent studies showed that B cells activated in vitro with enteroantigen (eAg) are capable of attenuating $CD4^+$ T cell transfer colitis when coinjected with the colitogenic T cells (19). The mechanism of suppression may involve B cell-mediated presentation to T cells of enteroantigenic peptides in a tolerogenic environment created by the co-injected, eAg activated B cells. In line with this observation, we also showed that eAg binding to the B cell Ig triggers IL-10 secretion (20) suggesting a mechanism by which eAg-activated B cells may control transfer colitis. Since eAg-activated B cells can be immortalized by cell hybridization (20), the aim of the present work was to develop a B cell/ hybridoma cloning strategy for eAg-activated B cell hybridomas which as such would be a valuable tool for future investigations of the role of eAg specific B cells in experimental colitis and in human IBD. In addition, we tested if these B cell hybridoma cells produce regulatory cytokines relevant for T cell immune modulation.

Materials and Methods

Animals and cells

Six weeks old female BALB/c mice were purchased from Taconic (Bomholtgaard, Denmark) and kept in the animal facility of the Panum Institute. Mice were sacrificed by cervical dislocation prior to dissection and preparation of cells from the spleen. Single cell suspension of cells were produced by gently pressing the spleen through a 70μm disposable nylon strainer (BD Falcon, Albertslund, Denmark, cat. no. 352350) followed by centrifugation. The cell pellet was re-suspended in RPMI1640 supplemented with glutamax (substrate division, Panum Institute), 10% heat inactivated fetal calf serum (FCS), 1% penicillin and streptomycin, and $5x10^{-5}$ M 2-mercaptoethanol (referred to as complete medium).

B cells

B cells were isolated from spleen cell suspensions with a standard kit (Stemcell Technologies from Cambridge, UK) according to the manufacturer's description. Purity of isolated B cells was >98% as confirmed by flow cytometry. The cells were reacted with enteroantigen (see below) at a protein concentration of 100 microgram/ml of culture medium for 3 days prior to the fusion experiments.

Enteroantigen (eAg) extract

Murine faecal extract was obtained as described previously by harvesting the faecal

matter from the colon and cecum of BALB/c mice (16) and the protein concentration was adjusted to 4 mg/ml and stored at -80° C.

Hybridoma culture and measurement of anti-eAg reactivity

CD19 cells purified from spleens were activated by exposure to eAg for three days. Activated CD19 cells and HAT-sensitive X63 lymphoma cells, at various cell ratios were fused using polyethylene glycol (PEG) 1500 (Roche, cat. no. 10783641001) as fusion agent (see Table 1).

Table 1. Exposure of X63 or eAg-activated CD19+ B cells affects viability

Cell type	Cell*/ml prior to fusion	Viable cells/ml, 10 min	Viable cells,/ml, 24 hrs
X63 fused	1.000.000	305.555	346.666
X63 not fused	1.000.000	1.108.000	1.676.666
CD19+ B cells fused	1.000.000	< 1000	< 1000
CD19+ B cells not fused	1.000.000	1.000.000	Not tested
CD19+ B cells fused	20.000.000	2.500.000	1.000.000

*Centrifuged cell pellet from 1 ml was fused

The fusion was performed over 1 min. in 800 microliter PEG under constant slow stirring followed by addition and stirring of 9 ml serum free RPMI-1640 medium over the next two minutes. This procedure was followed by centrifugation (1600 rpm) and resuspension in HAT containing medium (RPMI1640 + NaHCO3 + glutamax + 20% FCS + 2% 50x HAT (Gibco, cat. no.21060017) + 1% pen/strep and + 1% 0.1M NatriumPyrovat + 10ng/ml rhIL-6 + 10ng/ml rmIL-4). After fusion cells were cultured for 10 minutes, 24 hrs and 10 day followed by at limiting dilution (LD) culture in titrated cell numbers (see results) in the inner 60 wells of five 96 well culture plates. Ten days after the fusion the culture medium was replaced by HT culture medium (RPMI-1640 with 10% FCS, 1mM sodium pyruvate and 1% penicillin and streptomycin). At regular intervals, the plates were inspected through a microscope. In microcultures showing less than 30% growing cells (ie. considered LD growth), 100 microliter supernatant was recovered and tested for reactivity against enteroantigen using Maxisorp 96 well plates (Nunc, Roskilde,Denmark- cat. no. 44240421) which were coated with 2 microgram eAg/well. Peroxidase-conjugated rabbit anti-mouse Ig (Dako, Copenhagen, Denmark) was used as detection reagent. Supernatants from wells not containing growing cells were used as negative controls. Clones producing eAg binding antibody were considered positive when the absorption signal was higher than the mean of the control signal from wells with no cell growth + 4 SD values ($p<0.001$). To test the anti-eAg secreting stability of an established hybridome (D4, see results), the hybridoma cells were seeded at 2 cells per well, inspected with regular intervals, and when clonal growth was established the supernatants were tested for the presence of anti-eAg antibody. Again, clones producing

eAg binding were scored as mentioned above for LD clones. Two anti-eAg specific hybridomas, D4 and F8, were expanded in cell fabrics in accordance with the manufactures' protocol. Isotype testing of the Ig in the culture supernant was performed using mouse isotyping test kit.

Test of hybridoma eAg-specific proliferation and cytokine secretion

Hybridomas were pulsed overnight with eAg extract in complete medium. The hybridomas were cultered with eAg extract and was incubated at 37°C, 5% CO2 with addition of 3[H]-thymidine (0.5 μCi) for the last 18 hours. The cells were harvested and level of proliferation was determined as incorporated 3[H]-thymidine measured by liquid scintillation counting. Simultaneously, supernatants were harvested and assayed for Interferon (IFN)-γ, IL-10, IL-12p70, IL-1β, IL-6 and Tumor Necrosis Factor (TNF)-α using a multiplex kit from Mesoscale.

Table 2. Limiting dilution (LD) set up and clonality data from fusion of eAg activated CD19+ B cells and X63 lymphoma cells

Experiment no.	1	2	3	4
Time from fusion to LD culture	10 min	24 hrs	10 days	10 days
CD19:X63 fusion ratio	1:1*	10:1**	1:1*	3:1***
Cell no. per microculture well****	50.000	100	2.5	2,0
Percent clone frequency	0,0012	0.45	30.3	17,0
Percent anti-eAg pos. clones	0,0002	0,012	2.6	15,8

- *10^7 $CD19^+$ cells + 10^7 X 63 cells
- **$5x10^7$ $CD19^+$ cells + $5x10^6$ X63 cells
- ***$3x10^7$ $CD19^+$ cells + 10^7 X 63 cells
- ****Data obtained from titrated doses of fused cells. Clone frequency is calculated from at least 60 microcultures.

Results and discussion

Generation of B cell hybridomas

The toxic effect of the fusion medium polyethylene glycol (PEG) 1500 on X63 and eAg-activated CD19+ B cells and subsequent culture in HAT containing culture medium was tested on titrated cell numbers of eAg-activated X63 lymphoma cells and CD19+ B cells. As shown in Table 1, X63 cells are rather resistant to 1 min of concentrated PEG whereas eAg-activated CD19+ B cells appeared to be extremely sensitive to the toxic activity of PEG. Thus, PEG exposure of 10^6 eAg-activated CD19+ B cells resulted in > 99% loss of viable cells 10 min after PEG exposure. Exposure of 20 x 10^6 activated CD19+ B cells to PEG resulted in only 12.5% viable cells after 10 min declining only 5% after 24 hrs of culture. Thus, the 1 min fusion *per se* is extremely toxic to activated B cells. In contrast, after ten minutes exposure of 10^6

X63 cells to PEG, only half of the cells were dead, and 24 hrs later one third of the cells were still viable as judged by dye exclusion test.

Based on the data in Table 1, X63/CD19+ B cells were fused in PEG at cell ratios presented in table 2. Table 2 shows the results of 4 individual fusion experiments in which eAg-activated CD19+ B cells fused with X63 B lymphoma cells were titrated into micro culture wells either directly after fusion in numbers from 10.000 to 100.000 cells per well or they were grown as bulck cultures for 3 and 10 days, respectively, before being titrated into micro culture wells at cell numbers from 50 to 500 cells/well (cells from 3 days bulck culture) or 0.5 to 10/well (cells from 10 days bulk culture). Only clones from cell culture plates in which less than 30 of the 60 well culture plate contained growing cells were considered monoclonal.

Depending on the conditions used, the frequency of hybridoma cell clones and in particular of eAg-secreting clones varied considerably. Thus directly cloning of fused X63/CD19+ B cells in HAT medium give rise to the lowest clonal frequencies: 0.0012% total clones and 0.0002% eAg-reactive clones, respectively. However, culturing the fused cells for 24 hrs prior to subculture under limiting dilution conditions increased the frequency of total clones to 0.45% and eAg-reactive clones to 0.012%, respectively. Culturing the fused cells as bulck culture for 10 days before subcloning at limiting dilution conditions resulted in the highest frequency of clones, both total (17-30.3 %) and eAg-specific (2.6 – 15.8). In addition it appeared that by increasing the number of eAg-activated B cells during the fusion process increase the frequency of anti-eAg specific clones (experiment no.4). Thus it appears that bulk culture of fused cells prior to LD culture very significantly favour the growth of eAg-reactive clones.

Frequency and stability of cloned hybridomas.

Two hybridomas were established, D4 and F8 during the procedure described above. To test the anti-eAg secreting stability of the established hybridomas, the cells from one of the hybridomas, D4, was subcloned into 60 microwells in 10 plates at 2 cells per well. At day 14 of culture, out of 600 wells, total 224 wells were without cell growth, 366 wells contained cell growth alone without anti-eAg antibody in the culture supernatants whereas 35 wells with equal fine cell growth were positive for anti-eAg antibody also, Table 3. This means that the clonability of the subcloned hybridomas is high (approximate 60%) but only approximately 10 % of subcloned hybridoma cells have preserved anti-eAg antibody secretion.

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Table 3. The stability of subcloned D4 eAg-secreting hybridomas in ten microwell plates with each 60 wells.

	#Empty wells/60 wells	# Wells with eAg proliferation +antibody/60 wells	#Wells with eAg proliferation -antibody/60 wells
Plate 1	15	45	1
Plate 2	27	33	1
Plate 3	14	46	7
Plate 4	14	46	5
Plate 5	32	28	7
Plate 6	25	35	3
Plate 7	24	26	3
Plate 8	27	33	4
Plate 9	24	36	2
Plate 10	22	38	2
Total out of 600 wells	**224**	**366**	**35**

Table 4. eAg specific proliferation (cpm)

	Experiment 1				Experiment 2			
	No eAg	100µg/ml eAg	1µg/ml eAg	0.01 µg/ml eAg	No eAg	100µg/ml eAg	1µg/ml eAg	0.01 µg/ml eAg
D4 10.000 celler	575	705	658	594	705	879	864	717
D4 50.000 celler	1292	1563	1586	1314	1450	1704	1619	1584
F8 10.000 celler	295	431	400	332	408	589	562	436
F8 50.000 celler	1019	1186	1159	954	951	1160	1056	871
X63 10.000 celler	3656	5565	4752	4228	4437	4159	4200	3468
X63 50.000 celler	6687	8783	7013	7432	8180	8358	8258	7691

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eAg exposure stimulates hybridoma proliferation and cytokine secretion

From the limiting dilution cultures two hybridomes, D4 and F8, were further expanded in a cell fabric system (see Materials and Methods) and kept as cell cultures for an expanded time period. To see if these hybridomas are still sensitive to eAg-exposure, the cells were cultured with titrated concentrations of eAg for three days and the degree of proliferation was tested by 3H-Tdr incorporation. Table 4 shows a small but eAg-specific proliferation activation of B cell hybridomas as measured by proliferation which was dependent on eAg concentration and B cell hybridoma concentration. However, eAg exposure also appears to have some unspecific effect on X63 cells.

In addition, we tested the culture supernatants of eAg-exposed hybridoma cells for various cytokines. IL-10 was the only cytokine which could be detected above the limits for the detection system amongst the cytokines tested. Thus, an eventual secretion of IFN-γ, IL-12p70, IL-1β, IL-6 and TNF-α was below the detection levels of these cytokines (see supplementary table I). The results for IL-10 are shown in Table 5.

Supplementary Table I, Lower limits of cytokine and chemokine detection.

Cyto/chemokines	LLOD pg/ml
IFN-γ	0,2
IL-10	6,4
IL-12p70	10
IL-1β	0,44
IL-6	2,6
TNF-α	1

Table 5. IL-10 secretion (pg/ml) of hybridomas, D4 and F8, exposed to eAg

Cell	Cell number	Stimulus eAg µg/ml	Exp. 1 (n=2)*	Exp.2 (n=1)
D4	10.000	None	575	705
D4	10.000	100	705	879
D4	10.000	1	1292	864
D4	10.000	0.01	1586	717
D4	50.000	None	1292	1450
D4	50.000	100	1563	1704
D4	50.000	1	1586	1619
D4	50.000	0.01	1314	1554
F8	10.000	None	295	589
F8	10.000	100	431	589
F8	10.000	1	400	562
F8	10.000	0.01	332	436
F8	50.000	None	1019	951
F8	50.000	100	1186	1160
F8	50.000	1	1169	1056
F8	50.000	0.01	954	871

*Mean of two replicate cell cultures.

IL-10 secretion was cell-dose dependent and cells exposed to the lowest levels of eAg showed an increased activity of IL-10 secretion suggesting that high levels of eAg exhibit some kind of high-dose inhibition, typical for the Ig response of B cells to antigen exposure.

Conclusion

Recently, we demonstrated that co-transfer of eAg-exposed B cells ameliorate experimental colitis in the T cell transfer model of colitis (19). Generation of eAg-specific B cell hybridomas would be a unique tool to investigate the aberrant immune response towards eAg's in experimental colitis as well as in human IBD.

Here, we used a fusion medium with polyethylene glycol (PEG) to fuse X63 and in-vitro eAg-activated CD19+ B cells followed by subsequent culture in HAT containing culture medium. We were able to clone two eAg-specific B cell hybridomas which showed increased proliferation and IL-10 secretion in the presence of eAg.

In conclusion, eAg-activated B cells can be immortalized by cell hybridization and show potential for eAg-specific activation and production of regulatory cytokines. Such cells could be an important future tool for understanding the role of B cells in experimental colitis and human IBD.

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