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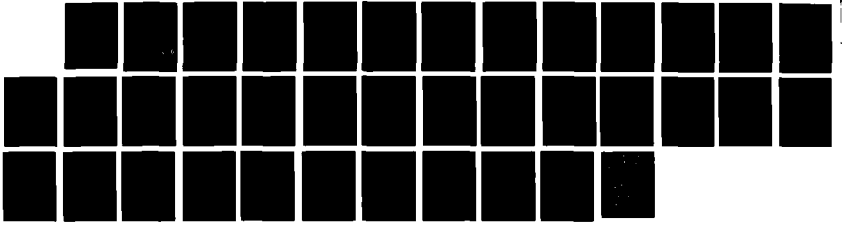

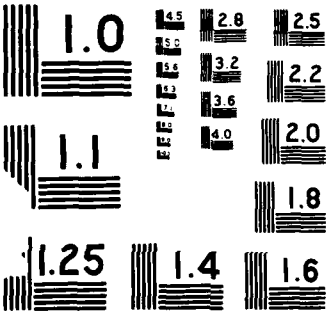
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Human Hybridomas for Exotic Antigens

Annual Report

Dr. Melvin Cohn, Ph.D.

January 1, 1989

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In this final year of our program of work we have investigated methods for in vitro induction of B cells from human peripheral blood and spleen using a prototype exotic antigen Keyhole limpet hemocyanin and for reference a prototype conventional antigen Diphtheria toxoid. To ensure that maximal induction would occur, various sources of growth and differentiation factors were added to cultures. An enhanced ELISA assay capable of detecting 1-10 ng/ml of specific antibody was employed, allowing us to detect the antibody secreted by 20-200 cells in a culture volume of 0.2 ml (~~10^3~~ antibody-producing cells secrete sufficient antibody in one day to reach 10 ng in one ml-or ~~10ng/ml~~). We also attempted to capture induced B cells at an early stage of fusion with the human tumor cell line WI-L2-729-HF_2. Despite extensive testing we were unable to reliably detect specific antibody secretion by any of the methods tested.

In conclusion we draw attention to the strong possibility that the immune system (whether in mice or humans) is structured in such a way that primary in vitro immune responses will be impractical due to the low frequency of inducible antigen-specific B cells in both peripheral blood and lymphoid organs. Keywords: Monoclonal antibodies. (AW)

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SUMMARY

In this final year of our contract to develop human hybridomas for exotic antigens we have focussed most of our effort on investigating techniques for in vitro immunization. For these experiments we primarily used peripheral blood lymphocytes obtained from normal healthy donors by lymphapheresis. The two standard antigens chosen to represent a primary and a secondary immune response were keyhole limpet hemocyanin and diphtheria toxoid respectively.

The experiments are broken down into 9 major groupings each of which represents a single large experiment started with a common pool of cells and carried through as a unit. This allowed us to distinguish between variation within experiments and variation between experiments. In general there was little variation within experiments and large variation between experiments. Analyzing these experiments involved screening over 10,000 culture supernatants for total immunoglobulin and specific antibodies. In some cases where cell growth in cultures looked particularly promising we fused the PBL with our standard reference tumor parent WI-L2-729-HF_2 to test hybridomas for antibody secretion.

No matter how the results are looked at they are clear and unequivocal – in no case could we reliably detect B cells secreting antibody from a primary in vitro immune response. Considering the wide range of conditions studied, and the collective results of others, we can quite reasonably conclude that, from a practical standpoint, in vitro immune B cell responses are so weak as to be of little significance. Since T cell responses can be regularly obtained, including responses in both the helper and killer classes, it is difficult to argue that there are too many suppressors, too many macrophages, and so on, in searching for an explanation of the lack of B cell responses. While acknowledging the unique features of human and murine immune systems, the dominant theme is that mice and men are not very different – evidence of primary in vitro immune responses is, in our opinion, hardly any better in mice than in man.

To reconcile our earnest desire to make in vitro immunization a practical technique with the apparent finality of the conclusions imposed by the data, we found a likely explanation in our discovery in an almost unrelated theoretical project that the humoral compartment of the immune system is modular in nature and subject to stringent limitations on the frequency of antigen specific B cells.

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FOREWORD

Citations of commercial organizations or trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Research Council (DHEW Publication No. (NIH)78-23, Revised 1987).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45FR46.

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1. OBJECTIVES.

The overall goal of the work being carried out under this contract is the development of methods for generating human hybridomas specific for highly toxic exotic antigens. Of primary interest is the development of methods that will enable human B lymphocytes to be immunized in vitro in a way suitable for fusion with the B–cell tumor WI-L2-729-HF$_2$, thereafter enabling the capture of stable antibody-producing hybridomas. Included in the broad aims of this contract is the production of human hybridomas from immune donors, particularly in the case of volunteers immunized with attenuated vaccines and known to be producing protective antibodies against viruses of major military significance.

2. BACKGROUND.

To induce resting B cells and obtain a population of proliferating antibody-producing cells requires antigen, T-cell help, and one or more regulatory lymphokines. The addition of antigen to lymphocyte cultures provides the initial selective signal that distinguishes particular antigen-specific B cells from all others. While antigen specific T cells normally provide the necessary help effect, such cells are sufficiently rare that this additional degree of antigen specificity is impractical. Allogeneic T cells have a high frequency of T cells able to recognize the MHC alloantigens on responding B cells. As we and others have repeatedly shown in the mouse system, such allogeneic T cells can help all B cells carrying the alloantigens. This we have termed abnormal help (to emphasize that it is not specific for the immunizing antigen—as distinct from normal antigen-specific help). Thus, in principle, antigen, B cells from one donor and irradiated (or mitomycin C-treated) cells from an unrelated (allogeneic) donor can be stimulated tc the stage at which induced B cells become susceptible to lymphokines. It has been these well established principles which guided our experimental protocols.

The study of in vitro immune responses with human lymphocytes is largely confined to T cell responses. The number of claims of primary in vitro antibody responses is large, but none have proven consistently useful to date. Despite this singular lack of success we felt that the use of allogeneic (abnormal) help and an ample supply of lymphokines might be sufficient to induce B cells to a stage of differentiation at which they could at least be captured as hybridomas. As this report will illustrate, we have been no more successful than others in achieving the elusive goal of inducing in vitro humoral antibody responses.

The experimental section of this report is brief as we condense the experimental protocols and data into a compact form. Given that no clear cut positive results were obtained, no need for detailed dissection and presentation of data is needed. Further, given the large amount of "housekeeping" required to setup, maintain, monitor, and analyze each experiment, it was not possible to carryout more than 10 experiments during the year; even this was difficult since we were short-handed one person for much of the period.

3. EXPERIMENTAL METHODS

3.A Fusion method

3.A.i Preparation of PEG:

Weigh 1 g of PEG (MW 4000 from EM Science) into a sterile glass tube and melt the PEG in a boiling water bath, then transfer to 56° water bath. Prepare fresh a 25% (v/v) solution of DMSO in RPMI 1640 at room temperature. Add 1 ml of 25% DMSO to 1 gm of molten PEG to make 50% (w/v) PEG and 12.5% (v/v) DMSO. Transfer the 50% PEG to a 37° water bath ready for use.

3.A.ii Preparation of cells:–

WI-L2-729-HF_2 tumor cells are harvested at $\approx 10^6$ cells per ml and washed twice in RPMI 1640. Lymphocytes recovered at various stages of stimulation were harvested, washed twice in RPMI 1640, and then counted prior to fusion.

3.A.iii Fusion:–

Using a 50 ml conical centrifuge tube, mix up to 2.5×10^7 lymphocytes and 2.5×10^7 WI-L2-729-HF_2 cells and pellet; the procedure can be scaled down to using as few as 10^6 of each cell type. To the pelleted cell mixture 2 ml of PEG/DMSO is added and gently swirled to get the cells into suspension. After 7 min at room temperature, over about 30 sec add 50 ml of RPMI 1640 and gently pellet the cells. Resuspend the pellet in 20 ml of R20F medium (or to about 2×10^6 total cells per ml) and incubate in 100 mm plastic tissue culture dishes overnight at 37° in an atmosphere of 5% CO_2 in air.

3.A.iv Preparation of R10F medium:–

To 90 ml of RPMI 1640 add:
1ml of 1M HEPES buffer
1 ml of 100 mM sodium pyruvate
1 ml of 100× nonessential amino acids
1 ml of Fungi-Bact (Irvine Scientific)
150 μl of 50 mg/ml gentamicin
2.5 ml of 80 mM glutamine
10 ml of FCS from pre-screened batches.
10 μl of 2-mercaptoethanol (0.5 M stock)
(note: R10H is prepared by substituting human A/B serum and the number represents the final percentage composition with respect to the indicated serum)

3.A.v Plating of hybridomas for selection in HAT:–

After overnight incubation, cells are plated at 2×10^5 cells per 0.1 ml in 96 well trays. Next day (48 hr post fusion) each well receives 0.1ml of 2× HAT. One week later wells are topped off with 1× HAT. Thereafter, no exchange of media occurs until clones are macroscopically visible, at which time supernatants are harvested and the wells refed with 1×HAT.

HAT is prepared from a 100× HT supplement (supplied by Whittaker M.A. Bioproducts) and 100× Aminopterin (Sigma) which is prepared by dissolving 1.76 mg of aminopterin in 100 ml of water.

3.A.vi Stimulation of lymphocytes in culture:–

3.A.vi.a Fetal calf, Human A/B and serum-free media

Fetal calf serum is pre-screened to support a high cloning efficiency of WI-L2-729-HF_2 and hybridomas; lot-to-lot variation is considerable and usually about 1 in 10 is suitable. Several different suppliers have been tested with no overall superiority of any one.

Human AB serum pools were obtained from Whittaker Bioproducts.

Serum-free media; Nutricyte serum-free media was obtained from J. Brooks Laboratories.

3.A.vi.b Reformalinized Pansorbin (abbreviated SAC).

As supplied by Calbiochem, Pansorbin is a 10% (w/v) suspension of heat inactivated and formalin fixed *S. aureus* (Cowan I strain) cells in PBS. To reduce the shedding of cell wall components, the reformalinization of Pansorbin has been recommended. This is accomplished by resuspending the pellet of a 5 ml aliquot of Pansorbin in 5 ml of 1.5% formaldehyde and incubating at room temperature for 1 hr. Cells are then washed twice in 50 ml of PBS and reconstituted to 5 ml (i.e., a 10% suspension). For stimulation of PBL, SAC is diluted to a final concentration of 0.1–0.01% as determined by titrating each batch.

3.A.vi.c Lymphocyte culture conditions.

Lymphocytes (PBL or spleen) are cultured in RPMI 1640 supplemented with 10% (v/v) serum, or in serum-free medium alone at concentrations ranging from 2–3 $\times 10^6$ per ml in plastic tissue culture dishes, tissue culture flasks, or in 24 well tissue culture plates at 37° with an atmosphere of 5% CO_2 in air. When appropriate, SAC, TCGF, rIL-2, 2-way MLR supernatant, BCGF, or soluble antigen was added. In experiments designed to further evaluate the effects of mononuclear phagocytes, adherent cells were removed by incubating cells on plastic tissue culture dishes or acid washed glass petri dishes in either a one-stage or two-stage adherence for various lengths of time at 37° and removing the non-adherent cells by means of gentle trituration, followed by a wash.

3.A.vii Screening of culture supernatants and hybrids for immunoglobulin isotypes:–

Plates of the 96 well type (Falcon #3911) are sensitized after removing static with a damp paper towel. A 1:200 dilution of goat anti-human kappa (supplied by TAGO or CALTAG), a 1:700 dilution of goat anti-human lambda (supplied by TAGO), or a 1:1000 dilution of goat anti-human IgM and goat anti-human IgG (supplied by CALTAG) in PBS-AE (phosphate buffered saline containing 0.0065% azide and 0.04% EDTA) is used to sensitize plates. The sensitization is carried out overnight at 4°. Plates are then washed thoroughly with deionized water, followed by one wash with PBS-AE plus 0.05% Tween. Plates were blocked by adding 0.1 ml of 0.1% gelatine (w/v) in PBS-AE to give a significantly lower background. Plates are then washed twice with deionized water, followed by one wash with PBS-AE plus 0.05% Tween.

To appropriately sensitized plates 45 μl of antibody (usually from culture supernatants) was added and plates allowed to stand overnight at 4°. Next morning plates are warmed to room temperature, the supernatants drained, and plates washed twice with deionized water, followed by one wash with PBS-AE plus 0.05% Tween, then allowed to drain.

After plates have bound antibodies present in the culture supernatants, 50 μl of appropriately diluted goat anti-human immunoglobulin that has been coupled with alkaline phosphatase is

added and the plates allowed to incubate for 3–4 hours at 37°. Plates are then washed twice with deionized water, then once with PBS-AE plus 0.05% Tween and allowed to drain.

To determine the amount of human immunoglobulin bound to the plates 50 μl of nitrophenyl phosphatase is added and the plates allowed to stand overnight at 4° if the amount of human antibody bound to the plates is moderate, or allowed to stand overnight at room temperature if the amounts are low. The amount of nitrophenyl released is measured photometrically using an automated plate reader (Dynatech) illuminated with 405 nm wavelength light.

The various goat anti-human immunoglobulins conjugated with alkaline phosphatase as purchased (TAGO or CALTAG) are diluted 1:20,000 in 1% BSA in PBS-AE. The nitrophenyl phosphate substrate is prepared as a 1 mg/ml solution in 0.05 M sodium bicarbonate buffer pH 8.0.

3.A.viii Screening for Diphtheria Toxoid (DT) and Keyhole limpet hemocyainin (KLH) binding:–

Essentially the same techniques as described for assaying immunoglobulins are used to assay the binding activities of culture supernatants to Diphtheria Toxoid from the Massachusetts Public Health Laboratories (1 μg/ml) and Keyhole Limpet Hemocyanin from Calbiochem (0.1 μg/ml) The amounts used to typically sensitize plates were determined using normal human sera as a positive control for anti-DT and immune mouse serum for KLH. To then further increase the sensitivity of these assays supernatants were removed and replaced with 40 μl of 0.2 mM NADP in 50 mM diethanolamine buffer pH 9.0 containing 1 mM $MgCl_2$ at room temperature in the dark for 20 min. Then 110 μl of alcohol dehydrogenase (0.4 mg/ml), diaphorase (2.1–2.4 units/ml) and p-iodonitrotetrazolium violet (0.278 mg/ml) in 25 mM phosphate buffer pH 7.2 for a further 20 min before adding 25 μl of 0.4 M HCl to stop the reaction. Under these conditions we could readily detect 10–100 pg of alkaline phosphatase or the equivalent of 1–10 ng/ml of antibody bound to the plates (anti-DT or anti-KLH in our case).

3.A.ix Human T cell growth factor (TCGF), B cell growth factor (BCGF), recombinant IL-2 (rIL-2), and 2-way mixed lymphocyte reaction (MLR) supernatants:–

TCGF and BCGF were commercially available (Cellular Products, Inc.) and of tissue culture grade that has been analyzed for its ability to stimulate proliferation of T or B cells. A typical recommended working concentration is 5–10%.

Recombinant IL-2 (rIL-2) was a gift of the Cetus Corporation.

The 2-way MLR supernatant was prepared by admixing 2×10^6 unfractionated PBL from each of two donors in 2 ml final volume complete media in 24 well tissue culture wells for 48 hours. Culture supernatants were then harvested, centrifuged, and filtered before use.

4. RESULTS.

The results presented in this section have been organized to reflect the nine separate experiments which encompass the bulk of the work carried out this year.

Experiment 88.1

PBL (donor 26) were cultured in 96 well round bottom plates with 10^5, 3×10^5, 5×10^5, 8×10^5, or 10^6 cells /ml in serum-free Nutricyte medium. mitomycin C-treated allogeneic donor cells were added where indicated to 1/5 or 1/2 the number of untreated responder cells present per 0.2 ml round bottom well. The data presented in Tables 1 and 2 were obtained from cultures containing 10^6 or 10^5 responder cells respectively. Supernatants from replicate cultures were taken and assayed on the days indicated for anti-DT activity; results are expressed as ELISA optical densities.

Table 1

		anti-DT on day			
allo cells	ng/ml DT	3	5	7	10
0	0	.288	.286	.341	.095
0	1	.305	.329	.461	.164
0	5	.046	.046	.046	.048
0	10	.044	.041	.047	.044
1/5	0	.131	.200	.112	.050
1/5	1	.223	.254	.348	.071
1/5	5	.046	.044	.047	.045
1/5	10	.044	.043	.044	.046
1/2	0	.052	.049	.045	.052
1/2	1	.093	.129	.120	.055
1/2	5	.047	.047	.052	.068
1/2	10	.044	.042	.045	.048

Table 2

		anti-DT on day			
allo cells	ng/ml DT	3	5	7	10
0	0	.252	.189	.094	.073
0	1	.389	.252	–	–
0	5	.047	.056	.083	.052
0	10	–	–	–	–
1/5	0	.096	.082	–	.064
1/5	1	.291	.231	.107	.060
1/5	5	.046	.055	.045	.052
1/5	10	.046	.053	.048	.051
1/2	0	.048	.067	.050	.061
1/2	1	.118	.076	.374	.055
1/2	5	.048	.052	.045	.048
1/2	10	.048	.049	.047	.046

Comments:

In cultures set up with 10^6 total cells per well (Table 1) there was a small accumulation of anti-DT over the first 7 days of culture when 1 ng/ml of DT was present. At higher doses of DT (5–10 ng/ml) there was an apparent decrease in detectable anti-DT. In this case the lower levels of anti-DT seen on day 3 can probably be explained by the very sensitive anti-DT assay that might have detected residual spontaneous secretion or the products of cell lysis and increasing amounts of DT antigen absorbed this background anti-DT activity. The addition of allogeneic cells tended to decrease the apparent anti-DT response under all conditions. Lowering the number of cells per well ten fold to 10^5 total (Table 2), reduced the overall response, although one culture well (day 7, 1 ng/ml DT, and 1/2 allogeneic cells) did appear to contain a rare clone of B cells producing anti-DT.

Experiment 88.2

In this experiment PBL from donor 25 were cultured in numbers ranging from 10^5 to 10^6 in 2 ml wells with 1/4 the number of allogeneic mitomycin C-treated PBL, and from 0–10 ng/ml DT. Supernatants were sampled every two days for 10 days and assayed for anti-DT activity. Despite indications from the previous experiment that fewer allogeneic cells might be more "helpful," and less suppressive, no significant anti-DT response was obtained (data not shown).

Experiment 88.3

Following the lack of success in obtaining responses with allogeneic cells where suppression seemed to be a major factor, we turned to the use of cell-free growth and differentiation factors. In this experiment we tested the effectiveness of unfractionated MLR supernatants on primary anti-KLH and secondary anti-DT responses using PBL from donor 27.

Cultures set up in 1 ml wells with 2×10^6 cells per ml as follows:–

grp	DT	KLH	MLR supe-A
1	0	0	0
2	0	0.1	0
3	0	0.5	0
4	0.1	0	0
5	0.5	0	0
6	0	0	5%
7	0	0	10%
8	0	0.1	5%
9	0	0.1	10%
10	0	0.5	5%
11	0	0.5	10%
12	0.1	0	5%
13	0.1	0	10%
14	0.5	0	5%
15	0.5	0	10%

groups 16–30 were repeats of 1–15 using MLR supe-B.

No antibody response was detectable (data not shown).

Experiment 88.4

Continuing the testing of cell growth and differentiation factors, this experiment tested the effectiveness of rIL-2 and IL-2 as well as pokeweed mitogen (PWM) factors induced in situ by PWM on the anti-DT response of PBL (donor 28).

Cultures were 1 ml each and 2×10^6 PBL set up as follows:–

grp	DT	PWM (μg)	rIL-2(units)	TCGF (%)
1	0	0	0	0
2	0	0	0	10
3	0	0	1	0
4	0	0	0	10
5	0	0	50	0
6	0	0	100	0
7	0	0.5	0	0
8	0	1	0	0
9	0	0.5	1	0
10	0	1	1	0
11	0	0.5	10	0
12	0	1	10	0
13	0	0.5	50	0
14	0	1	50	0
15	0	0.5	100	0
16	0	1	100	0
17	0	0.5	0	10
18	0	1	0	10
19	0.5	0	1	0
20	1	0	1	0
21	0.5	0	10	0
22	1	0	10	0
23	0.5	0	50	0
24	1	0	50	0
25	0.5	0	100	0
26	1	0	100	0
27	0.5	0	0	10
28	1	0	0	10
29	0.5	0.5	0	0
30	1	1	0	0
31	0.5	1	0	0
32	1	0.5	0	0
33	0.5	0.5	0	10
34	1	1	0	10
35	0.5	1	0	10
36	1	0.5	0	10
37	0.5	0.5	1	0
38	1	1	1	0
39	0.5	0.5	100	0
40	1	1	100	0

No anti-DT responses were detectable in any group (data not shown).

Experiment 88.5

In this experiment TCGF and BCGF were added separately and in combination with two different allogeneic MLR supernatants (labelled D and F) or allogeneic cells. The PBL were obtained from donor number 27.

The following groups were set up:–

grp	DT(ng/ml)	MLR (%)	BCGF	TCGF	repeats
1D	0	0	0	0	40F
2D	0	0	0	10	
3D	0	0	10	0	
4D	0	10	0	0	
5D	1	0	0	0	
6D	0.5	0	0	0	
7D	1	0	5	5	19D
8D	0.5	0	5	5	20D
9D	1	0	0	10	
10D	0.5	0	0	10	
11D	1	0	10	0	15D
12D	0.5	0	10	0	16D
13D	1	10	0	0	9D
14D	0.5	10	0	0	10D
21D	1	1	1	1	43F
22D	0.5	1	1	1	44F
23D	1	2	2	2	45F
24D	0.5	2	2	2	46F
25D	0	1	1	1	
26D	0	2	2	2	
27D	0	0	5	5	
28	0	0	0	0	allo cells
29	0	0	0	10	allo cells
30	0	0	10	0	allo cells
31	1	0	0	0	allo cells
32	0.5	0	0	0	allo cells
33	1	0	0	10	allo cells
34	0.5	0	0	10	allo cells
35	1	0	10	0	allo cells
36	0.5	0	10	0	allo cells
37	1	0	5	5	allo cells
38	0.5	0	5	5	allo cells

Comments:

Although there was some fluctuation in the levels of IgM and anti-DT, no consistent pattern of response was detected (data not shown). There was, however, a marked effect of adding BCGF as seen by inspection of the cultures which s'owed owedgreatly increased numbers of both blasts and total cells.

Experiment 88.6

In this experiment we specifically tracked cell proliferation, blasting and total immunoglobulin production in addition to measuring anti-DT and anti-KLH activity (donor 27).

Cells were cultured in either 96 well ROUND bottom (Table 3) or FLAT bottom (Table 4) plates.

Table 3

			day 2	day 4			day 7			day 9		
DT	SAC*	BCGF	Ig	cells	blasts	Ig	cells	blasts	Ig	cells	blasts	Ig
–	–	–	1.57	8.3	20	1.48	4.2	33	1.73	4.5	50	1.75
–	–	5%	1.45	10.0	26	1.48	10.2	61	>2	10.6	55	>2
–	–	10%	1.52	7.2	36	1.53	11.6	79	>2	13.4	60	>2
–	1x	–	1.37	4.4	36	1.27	1.2	17	1.49	1.4	11	1.49
–	1x	5%	1.38	3.8	47	1.43	5.0	80	1.68	6.8	79	1.8
–	1x	10%	1.36	2.6	46	1.29	10.4	92	>2	8.8	95	1.96
–	2x	–	1.42	6.4	19	1.41	2.2	45	1.93	6.2	71	>2
–	2x	5%	1.38	6.4	34	1.34	8.6	93	>2	15.4	90	>2
–	2x	10%	1.41	5.0	44	1.73	17.0	98	>2	12.0	75	>2
0.5	–	–	1.38	3.7	46	1.56	3.8	53	1.94	1.8	56	1.68
1.0	–	–	1.37	7.2	22	1.51	3.6	56	1.81	3.8	63	1.81
0.5	–	5%	1.38	6.2	26	1.56	6.0	73	1.73	5.0	60	1.77
0.5	–	10%	1.29	7.4	30	1.62	6.4	53	1.78	4.8	50	1.0
1.0	–	5%	1.37	7.8	36	1.59	4.7	61	1.86	4.2	48	1.32
1.0	–	10%	1.44	7.2	22	1.56	4.8	71	1.94	5.4	44	1.67
0.5	1x	5%	1.41	10.6	36	1.39	2.8	71	1.74	2.4	50	1.56
0.5	1x	10%	1.43	3.6	22	1.63	4.3	69	1.91	3.0	80	1.52
0.5	2x	5%	1.41	5.8	17	1.27	3.2	69	1.91	3.6	61	1.70
0.5	2x	10%	1.46	4.2	24	1.45	4.6	78	1.90	3.6	83	1.75
0.5	1x	–	1.38	4.4	14	1.32	2.2	27	1.63	1.6	13	1.26
0.5	2x	–	1.36	6.0	20	1.47	3.0	13	1.77	3.4	29	1.65
1.0	1x	5%	1.37	2.2	27	1.53	3.2	56	1.74	5.0	88	1.73
1.0	1x	10%	1.32	3.0	27	1.57	3.0	67	1.73	3.6	94	1.50
1.0	2x	5%	1.26	3.4	2.4	1.48	2.2	64	1.71	2.8	64	1.70
1.0	2x	10%	1.35	1.0	40	1.55	2.8	71	1.78	3.8	58	1.63
1.0	1x	–	1.38	1.4	29	1.53	2.2	9	1.46	2.4	8	1.39
1.0	2x	–	1.45	1.8	22	1.48	2.4	13	1.71	2.6	23	1.88

* 1x = 0.1% SAC; 2x = 0.01% SAC

Cells expressed as number multiplied by 10^{-5}. All groups were assayed for anti-DT activity and none were found positive above background levels. Blasts are expressed as a percentage of the total cells and Ig (immunoglobulin) data represents the ELISA optical densities using a polyvalent anti-immunoglobulin which measures IgG and IgM.

Table 4

			day 2			day 4			day 7			day 9	
DT	SAC*	BCGF	Ig	cells	blasts	Ig	cells	blasts	Ig	cells	blasts	Ig
–	–	–	1.55	4.4	9	1.57	4.2	43	1.77	3.4	53	>2
–	–	5%	1.52	5.6	21	1.57	12.2	84	>2	10.4	81	>2
–	–	10%	1.51	5.6	28	1.63	10.0	86	>2	14.6	96	>2
–	1x	–	1.42	4.0	20	1.40	2.4	33	1.84	1.6	13	1.55
–	1x	5%	1.39	4.2	24	1.40	3.6	83	1.91	15.4	99	>2
–	1x	10%	1.41	1.8	44	1.54	14.0	96	1.97	19.6	100	>2
–	2x	–	1.43	3.4	24	1.49	2.0	50	1.91	3.0	47	2.0
–	2x	5%	1.32	3.0	40	1.59	9.0	91	>2	17.6	99	>2
–	2x	10%	1.28	3.2	69	1.57	15.0	100	>2	7.2	89	>2
0.5	–	–	1.52	7.2	14	1.58	5.0	44	1.71	3.8	58	1.71
1.0	–	–	1.43	9.0	22	1.63	3.5	41	1.59	3.2	38	1.75
0.5	–	5%	1.34	4.6	9	1.61	5.6	50	1.74	2.6	38	1.84
0.5	–	10%	1.39	3.4	18	1.62	5.6	57	1.73	2.2	45	1.54
1.0	–	5%	1.42	4.2	19	1.53	4.6	57	1.68	3.2	56	1.76
1.0	–	10%	1.47	4.0	20	1.57	3.8	53	1.73	2.2	45	1.54
0.5	1x	5%	1.36	3.8	11	1.39	2.4	92	1.64	3.8	95	1.76
0.5	1x	10%	1.21	2.4	50	1.46	3.6	78	1.63	4.0	90	1.74
0.5	2x	5%	1.25	4.6	22	1.51	3.4	47	1.89	4.8	67	1.86
0.5	2x	10%	1.38	3.4	47	1.44	5.4	70	1.84	4.8	88	1.80
0.5	1x	–	1.39	4.4	14	1.44	2.0	10	1.50	2.2	9	1.46
0.5	2x	–	1.39	3.8	16	1.46	4.4	9	1.59	1.8	44	1.66
1.0	1x	5%	1.45	2.6	23	1.48	2.6	54	1.51	3.2	75	1.66
1.0	1x	10%	1.38	2.2	36	1.50	3.2	81	1.49	2.4	67	1.65
1.0	2x	5%	1.48	3.8	26	1.54	3.0	53	1.82	6.0	77	1.84
1.0	2x	10%	1.50	2.6	31	1.59	3.8	68	1.74	3.0	67	1.64
1.0	2x	–	1.56	3.8	11	1.50	3.2	6	1.55	2.4	8	1.45
1.0	2x	–	1.42	3.6	17	1.40	3.2	19	1.57	2.4	25	1.58

* 1x = 0.1% SAC; 2x = 0.01% SAC

Cells expressed as number multiplied by 10^{-5}. All groups were assayed for anti-DT activity and none were found positive above background levels. Blasts are expressed as a percentage of the total cells and Ig (immunoglobulin) data represents the ELISA optical densities using a polyvalent anti-immunoglobulin which measures IgG and IgM.

Comments:

The results summarized in Tables 3 and 4 show a comparison between round and flat bottomed culture vessels. One notable feature of these results is the close similarity between the two sets of results, indicating that the shape of the culture vessel is not a significant factor, and more importantly that a given pool of cells and reagents plated and maintained as replicate pairs give essentially the same results—a strong demonstration that the variability we observe between experiments is due to factors other than those involved in setting up, maintaining, sampling, and assaying the cultures.

Experiment 88.7

In this experiment the effect of mitomycin C-treated allogeneic cells was reevaluated in conjunction with the purified growth factors TCGF and BCGF added as 10% (v/v) supplements to the cultures; in addition we tested possible effects due to the time of addition of growth factors and antigen (DT) which was added at 1 or 5 ng/ml final concentration. PBL (donor 27) were added at 6.4×10^5 per well in 96 well flat bottom microwell plates; each group was set up in twelve-fold replicas ready for harvesting supernatants every 2 days up to 24 days.

The following three tables outline the experimental groups:–

Table 5

grp	DT d0	BCGF d0	TCGF d0	SAC d0
1	0	0	0	0
2	0	0	10	0
3	0	10	0	0
4	1	0	0	0
5	5	0	0	0
6	1	0	10	0
7	5	0	10	0
8	1	10	0	0
9	5	10	0	0
10	1	5	5	0
11	5	5	5	0

Table 6

grp	DT d0	BCGF d3	TCGF d3	SAC d0
12	0	10	0	0
13	0	0	10	0
14	1	10	0	0
15	5	10	0	0
16	1	0	10	0
17	5	0	10	0
18	1	5	5	0
19	5	5	5	0
20	0	o	0	1x
21	0	0	0	2x
22	0	0	10	1x
23	0	0	10	2x
24	0	10	0	1x
25	0	10	0	2x
26	1	0	0	1x
27	5	0	0	1x
28	1	0	0	2x
29	5	0	0	2x
30	1	0	10	1x
31	5	0	10	1x
32	1	0	10	2x
33	5	0	10	2x
34	1	10	0	1x
35	5	10	0	1x
36	1	10	0	2x
37	5	10	0	2x
38	0	5	5	1x
39	0	5	5	2x
40	1	5	5	1x
41	5	5	5	1x
42	1	5	5	2x
43	5	5	5	2x

1x = 0.1% SAC; 2x = 0.01% SAC.

Table 7

grp	DT d3	BCGF d3	TCGF d3	SAC d0
44	1	0	0	0
45	5	0	0	0
46	1	0	0	1x
47	5	0	0	1x
48	1	0	0	2x
49	5	0	0	2x
50	1	0	10	1x
51	5	0	10	1x
52	1	0	10	2x
53	5	0	10	2x
54	1	10	0	1x
55	5	10	0	1x
56	1	10	0	2x
57	5	10	0	2x
58	1	5	5	1x
59	5	5	5	1x
60	1	5	5	2x
61	5	5	5	2x

1x = 0.1% SAC; 2x = 0.01% SAC.

There was no detectable anti-DT response in any experimental groups although total IgM levels were comparable to those found in Experiment 88.6.

Experiment 88.8

In this experiment we tested the response of spleen cells with various combinations of SAC, antigen and growth factors added at various times. Results in the following tables are the ELISA optical densities obtained with the various assays. The in vitro culture system used RPMI supplemented with 3% FCS and cells were seeded at 10^6 per well in 0.2 ml flat bottom wells.

Table 8

group 1: cells only

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.315	.407	.500	.409	.610	.750
lambda	.340	.308	.430	.420	.510	.480
IgM	.252	.346	.498	.500	.391	.463
IgG	.250	.340	.360	.370	.370	.300
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 9

group 2: 0.01% SAC only day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.083	.240	.370	.442	.614	.750
lambda	.190	.280	.340	.370	.430	.470
IgM	.174	.253	.448	.425	.385	.507
IgG	.320	.300	.360	.230	.360	.360
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 10

group 3: 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.160	.500	.820	.840	.570	.500
lambda	.490	.490	.500	.540	.480	.470
IgM	.247	.277	.776	1.16	1.26	1.30
IgG	.330	.360	.300	.330	.330	.360
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 11

group 4: 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.300	.400	.600	.700	.700	.600
lambda	.450	.410	.430	.470	.430	.460
IgM	.303	.381	1.27	1.53	1.64	1.50
IgG	.310	.290	.280	.370	.400	.380
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 12

group 5: 10 ng/ml DT day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.183	.305	.467	.468	.473	.422
lambda	.300	.290	.370	.420	.410	.410
IgM	.207	.270	.360	.420	.420	.440
IgG	.350	.350	.310	.380	.330	.250
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 13

group 6: 50 ng/ml DT day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.184	.189	.464	.424	.398	.411
lambda	.240	.270	.300	.380	.320	.320
IgM	.190	.290	.410	.440	.420	.430
IgG	.300	.310	.310	.310	.260	.220
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 14

group 7: 500 ng/ml DT day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.334	.300	.393	.460	.400	.300
lambda	.230	.310	.420	.380	.340	.320
IgM	.210	.290	.440	.400	.430	.430
IgG	.300	.350	.350	.320	.320	.300
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 15

group 8: 1 μg/ml DT day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.170	.274	.355	.400	.453	.370
lambda	.240	.260	.340	.350	.290	.310
IgM	.270	.280	.440	.440	.430	.420
IgG	.360	.290	.240	.280	.270	.250
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 16

group 9: 10 ng/ml DT + 0.01% SAC day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.321	.417	.513	.800	.800	.700
lambda	.260	.290	.390	.540	.420	.480
IgM	.180	.200	.400	.520	.500	.510
IgG	.330	.410	.420	.400	.350	.330
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 17

group 10: 50 ng/ml DT + 0.01% SAC day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.240	.260	.560	.700	.740	.670
lambda	.290	.240	.440	.380	.410	.330
IgM	.150	.210	.410	.410	.430	.370
IgG	.340	.340	.350	.390	.390	.310
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 18

group 11: 500 ng/ml DT + 0.01% SAC day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.220	.390	.840	.820	.820	.810
lambda	.170	.270	.490	.430	.510	.460
IgM	.161	.176	.457	.446	.472	.460
IgG	.380	.420	.440	.440	.400	.380
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 19

group 12: 1 μg/ml DT + 0.01% SAC day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.230	.370	.437	.560	.420	.531
lambda	.250	.320	.410	.410	.430	.370
IgM	.163	.213	.312	.416	.378	.364
IgG	.410	.370	.360	.370	.370	.280
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 20

group 13: 10 ng/ml DT + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.120	.130	.500	.500	.620	.520
lambda	.420	.340	.480	.620	.580	.570
IgM	.180	.230	.664	1.00	1.10	1.13
IgG	.470	.400	.330	.340	.330	.310
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 21

group 14: 50 ng/ml DT + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.260	.400	.560	.510	.620	.520
lambda	.540	.460	.480	.510	.450	.420
IgM	.210	.250	.680	.960	1.00	1.00
IgG	.400	.400	.310	.350	.350	.370
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 22

group 15: 500 ng/ml DT + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.100	.000	.200	.480	.410	.480
lambda	.420	.450	.510	.590	.490	.470
IgM	.260	.250	.730	1.02	1.11	1.14
IgG	.450	.461	.330	.350	.280	.270
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 23

group 16: 1 μg/ml DT + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.230	.140	.400	.560	.470	.460
lambda	.450	.460	.410	.460	.380	.320
IgM	.224	.261	.644	.944	1.00	.96
IgG	.360	.350	.270	.390	.380	.370
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 24

group 17: 10 ng/ml DT + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.130	.100	.180	.300	.410	.170
lambda	.380	.410	.210	.370	.290	.280
IgM	.220	.300	.960	1.35	1.37	1.35
IgG	.250	.250	.280	.350	.410	.410
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 25

group 18: 50 ng/ml DT + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.01	.100	.180	.300	.410	.170
lambda	.360	.320	.330	.330	.330	.300
IgM	.329	.290	.950	1.26	1.26	1.16
IgG	.310	.280	.320	.380	.410	.410
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 26

group 19: 500 ng/ml DT + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.100	.260	.240	.370
lambda	.420	.330	.350	.360	.290	.428
IgM	.210	.240	.960	1.30	1.35	1.40
IgG	.380	.260	.320	.430	.430	.430
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 27

group 20: 1 μg/ml DT + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.300	.210	.210	.330	.130	.190
lambda	.330	.240	.240	.320	.280	.190
IgM	.290	.300	.910	1.28	1.26	1.16
IgG	.250	.270	.290	.360	.350	.350
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 28

group 21: 10 ng/ml DT + 0.01% SAC day 0, 5% TCGF day 0 + 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.090	.000	.130	.270	.390	.190
lambda	.390	.300	.370	.340	.350	.430
IgM	.163	.165	.510	.920	1.12	1.25
IgG	.300	.360	.260	.300	.310	.350
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 29

group 22: 50 ng/ml DT + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.150	.230	.120	.170
lambda	.350	.290	.180	.180	.190	.170
IgM	.163	.180	.580	.930	1.04	1.04
IgG	.290	.310	.260	.320	.330	.340
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 30

group 23: 500 ng/ml DT + 0.01% SAC + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.050	.150	.030
lambda	.310	.320	.270	.170	.280	.350
IgM	.150	.207	.570	.990	1.14	1.12
IgG	.310	.310	.240	.280	.320	.280
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 31

group 24: 1 μg/ml DT + 0.01% SAC + 5% TCGF day 0, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.020	.000	.000	.000	.000
lambda	.340	.290	.260	.280	.210	.180
IgM	.180	.204	.460	.803	.960	.910
IgG	.280	.290	.230	.250	.230	.260
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 32

group 25: 10 ng/ml DT + 0.01% SAC + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.080	.080	.280	.390	.340	.250
lambda	.530	.400	.480	.440	.450	.350
IgM	.160	.200	.840	1.32	1.25	1.27
IgG	.230	.220	.270	.360	.360	.400
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 33

group 26: 50 ng/ml DT + 0.01% SAC + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.100	.120	.060	.320	.200	.280
lambda	.360	.270	.250	.270	.270	.260
IgM	.170	.190	.670	1.10	1.16	1.15
IgG	.200	.210	.240	.250	.250	.240
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 34

group 27: 500 ng/ml DT + 0.01% SAC + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.040	.120	.060	.310	.160	.120
lambda	.450	.480	.430	.370	.280	.360
IgM	.160	.210	.750	1.10	1.30	1.25
IgG	.300	.260	.290	.320	.300	.280
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 35

group 28: 1 μg/ml DT + 0.01% SAC + 5% TCGF + 5% BCGF day 0

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.093	.080	.020
lambda	.320	.320	.340	.380	.380	.380
IgM	.200	.222	.510	1.10	1.14	1.10
IgG	.180	.230	.190	.210	.280	.230
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 36

group 29: 10 ng/ml DT + 5% TCGF day 0, 0.01% SAC day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.000	.000	.000
lambda	.450	.430	.390	.370	.400	.430
IgM	.140	.170	.380	.810	.970	1.10
IgG	.330	.320	.240	.220	.230	.280
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 37

group 30: 50 ng/ml DT + 5% TCGF day 0, 0.01% SAC day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.000	.030	.020
lambda	.290	.240	.180	.240	.290	.260
IgM	.160	.150	.330	.720	.870	.850
IgG	.240	.260	.150	.140	.160	.240
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 38

group 31: 500 ng/ml DT + 5% TCGF day 0, 0.01% SAC day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.000	.050	.000
lambda	.440	.410	.340	.290	.270	.250
IgM	.140	.140	.390	.650	.860	.960
IgG	.410	.330	.260	.230	.320	.360
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 39

group 32: 1 μg/ml DT + 5% TCGF day 0, 0.01% SAC day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.000	.000	.000
lambda	.230	.330	.180	.190	.170	.160
IgM	.240	.210	.260	.400	.680	.830
IgG	.310	.230	.140	.160	.250	.260
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 40

group 33: 5% TCGF + 0.01% SAC day 0, 10 ng/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.030	.130	.050	.370	.600	.520
lambda	.330	.250	.280	.430	.460	.470
IgM	.140	.150	.390	.880	1.19	1.25
IgG	.350	.360	.290	.300	.450	.470
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 41

group 34: 5% TCGF + 0.01% SAC day 0, 50 ng/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.020	.110	.280	.450	.370	.290
lambda	.130	.210	.300	.340	.370	.270
IgM	.120	.150	.430	.870	1.10	1.02
IgG	.360	.350	.300	.300	.350	.370
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 42

group 35: 5% TCGF + 0.01% SAC day 0, 500 ng/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.060	.120	.070	.140	.290	.160
lambda	.330	.360	.400	.380	.410	.460
IgM	.140	.140	.290	.500	.920	.990
IgG	.360	.330	.340	.300	.510	.330
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 43

group 36: 5% TCGF + 0.01% SAC day 0, 1 μg/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.160	.120	.450	.550	.450	.480
lambda	.380	.300	.290	.350	.360	.300
IgM	.200	.190	.530	.850	1.00	1.10
IgG	.340	.340	.290	.280	.360	.240
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 44

group 37: 5% TCGF day 0, 0.01% SAC + 10 ng/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.200	.020	.230	.250	.210	.300
lambda	.450	.330	.360	.370	.270	.230
IgM	.160	.160	.380	.630	1.10	1.12
IgG	.350	.300	.260	.310	.450	.410
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 45

group 38: 5% TCGF day 0, 0.01% SAC + 50 ng/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.130	.020	.010	.200	.220	.200
lambda	.170	.250	.200	.230	.240	.270
IgM	.190	.180	.510	.750	.820	.830
IgG	.360	.300	.180	.230	.240	.350
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 46

group 39: 5% TCGF day 0, 0.01% SAC + 500 ng/ml DT day 1, 5% BCGF day 2

assay	day 13	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.010	.300	.150	.300
lambda	.240	.390	.230	.190	.320	.340
IgM	.190	.170	.430	.880	1.09	1.14
IgG	.460	.410	.360	.440	.560	.550
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Table 47

group 40: 5% TCGF day 0, 0.01% SAC + 1 μg/ml DT day 1, 5% BCGF day 2

assay	day 1	day 2	day 5	day 7	day 9	day 12
kappa	.000	.000	.000	.090	.100	.000
lambda	.090	.220	.110	.220	.140	.100
IgM	.360	.140	.300	.590	.730	.830
IgG	.400	.330	.250	.230	.330	.290
anti-DT	–	–	–	–	–	–
anti-KLH	–	–	–	–	–	–

Comments:

Groups 1 and 2 represent controls with either no additions, or SAC alone and together they form a baseline comparison. The addition of TCGF and BCGF (groups 3 and 4) resulted in a sharp increase in total immunoglobulin after day 5 of culture. When increasing amounts of DT (from 10 ng/ml to 1 μg/ml) was added (groups 5–8), there was a small but consistent decrease in total immunoglobulin although κ antibodies tended to be more reduced than either λ, IgM, or IgG. The addition of SAC with DT (groups 9–12) reversed the decrease in immunoglobulin except at the highest concentration of DT. In groups 13–16 5% TCGF and DT were added on day 0 and 5% BCGF on day 2; this resulted in a response pattern similar to that obtained with TCGF and BCGF in the absence of DT. However, when DT, TCGF and BCGF were added on day 0 (groups 17–20), there was a marked decrease in the levels of κ late in the response. Adding SAC, DT and TCGF on day 0 and BCGF on day 2 (groups 21–24) resulted in the virtual abrogation of the κ antibodies without affecting the overall levels of IgM, IgG, or λ.

Comparing groups 21–24 with groups 13–16, the addition of SAC on day 0 made the loss of κ antibodies more acute whereas SAC and DT alone (groups 9–12) had virtually no effect on the κ response as seen with DT alone (groups 5–8). Two elements appear important in this inhibition of the κ response; the presence of DT and the 2 day delay in adding BCGF. The importance of timing is further seen in groups 25–28 where DT, SAC, TCGF, and BCGF were all added at the beginning of the culture; the suppression of κ responses is less marked though nonetheless significant. In groups 29–32 the κ response is again abrogated when DT and TCGF are added on day 0, SAC on day 1, and BCGF on day 2. Adding SAC and TCGF on day 0, DT on day 1, and BCGF on day 2 seemed to delay the suppression of the κ response (groups 33–36). Finally in groups 37–40 where TCGF was added on day 0, SAC and DT on day 1, and BCGF on day 2 resulted in both a delay in appearance of the κ suppression and a dose dependent effect of DT such that at higher doses of DT the suppression became more marked until at 1 μg/ml the κ response was again abrogated.

Experiment 88.9

Induction of DT specific antibody by in vitro immunization of PBL

Cells from donor 31 assayed either whole population or after removing the bulk of adherent cells. Selected groups of cultures of nonadherent cells were fused with WI-L2-729-HF_2 on days 3 and 7 in an attempt to determine if hybridomas could be captured and tested for antibody secretion.

Table 48 illustrates the conditions used with whole PBL population (2.3×10^6 cells per 1.5 ml in 2 ml wells; Table 49 is essentially the same but using nonadherent PBL is similar culture vessels.

Table 48

grp	DT	KLH	BCGF + TCGF	BCGF	0.01% SAC
1	0	0	0	0	0
2	0	0	0	0	+
3	0	0	0	10	0
4	0	0	+	0	0
5	7	0	0	10	0
6	33	0	0	10	0
7	330	0	0	10	0
8	700	0	0	10	0
9	0	7	0	10	0
10	0	33	0	10	0
11	0	330	0	10	0
12	0	700	0	10	0
13	7	O	0	0	+
14	33	0	0	0	+
15	330	0	0	0	+
16	700	0	0	0	+
17	0	7	0	0	+
18	0	33	0	0	+
19	0	330	0	0	+
20	0	700	0	0	+
21	7	0	+	0	0
22	33	0	+	0	0
23	330	0	+	0	0
24	700	0	+	0	0
25	0	7	+	0	0
26	0	33	+	0	0
27	0	330	+	0	0
28	0	700	+	0	0
29	7	0	0	10	+
30	33	0	0	10	+
31	330	0	0	10	+
32	700	0	0	10	+
33	0	7	0	10	+
34	0	33	0	10	+
35	0	330	0	10	+
36	0	700	0	10	+
37	7	0	+	0	+
38	33	0	+	0	+
39	330	0	+	0	+
40	700	0	+	0	+
41	0	7	+	0	+
42	0	33	+	0	+
43	0	330	+	0	+
44	0	700	+	0	+

45 TO 52 REPEATS

Table 49

grp	DT	KLH	BCGF + TCGF	BCGF	0.01% SAC
61	0	0	0	0	0
62	0	0	0	0	+
63	0	0	0	10	0
64	0	0	+	0	+
65	5	0	0	10	0
66	25	0	0	10	0
67	250	0	0	10	0
68	500	0	0	10	0
69	0	5	0	10	0
70	0	25	0	10	0
71	0	250	0	10	0
72	0	500	0	10	0
73	5	0	0	0	+
74	25	0	0	0	+
75	250	0	0	0	+
76	500	0	0	0	+
77	0	5	0	0	+
78	0	25	0	0	+
79	0	250	0	0	+
80	0	500	0	0	+
81	5	0	+	0	0
82	25	0	+	0	0
83	250	0	+	0	0
84	500	0	+	0	0
85	0	5	+	0	0
86	0	25	+	0	0
87	0	250	+	0	0
88	0	500	+	0	0
89	5	0	0	10	+
90	25	0	0	10	+
91	250	0	0	10	+
92	500	0	0	10	+
93	0	5	0	10	+
94	0	25	0	10	+
95	0	250	0	10	+
96	0	500	0	10	+
97	5	0	+	0	+
98	25	0	+	0	+
99	250	0	+	0	+
100	500	0	+	0	+
101	0	5	+	0	+
102	0	25	+	0	+
103	0	250	+	0	+
104	0	500	+	0	+

105-109 repeat 100

Although no cultures or hybridomas were found to secrete anti-DT or anti-KLH, we did find that a very high fusion efficiency could be obtained from 3 day cultures stimulated with either BCGF alone or a mixture of BCGF and TCGF (Table 50), but by day 7 the cultures were unable to form stable hybrids (Table 51).

Table 50

Cultures harvested on day 3

grp	blasts (%)	cells $\times 10^{-6}$	comments
65	43	1.5	all +
68	31	1.5	all +; < grp 65
69	52	1.6	not so good
72	45	1.4	all +
81	40	1.3	all +
84	39	1.0	all +; multiple
85	44	1.3	highest; > grp 88
88	36	1.3	all +; multiple
89	37	1.0	all +
92	36	1.3	all + > grp 89
93	18	0.6	all +
96	25	0.8	all +; < grp 93
97	19	1.0	all +
100	31	0.7	half +
101	24	1.3	all +; > grp 104
104	32	1.3	all +

Table 51

cultures harvested on day 7

grp	blasts (%)	cells $\times 10^{-6}$	comments
82	44	7.0	no hybrids
83	65	4.4	no hybrids
86	96	6.0	no hybrids
87	95	4.4	no hybrids
98	93	2.0	no hybrids
99	95	2.0	no hybrids
102	98	4.0	no hybrids
103	99	5.0	no hybrids

Cells recovered from three or seven day cultures (cell totals are given in each table above) were fused with WI-L2-729-HF_2 at a 1:1 ratio and plated in 96 wells so that each well received on average 10^4 cultured cells. For each well to show growth of hybrids the fusion efficiency had to be better than 10^4, and in those cases where every well had multiple clones the fusion efficiency would have been closer to 10^3.

Although we were able to repeat the essential finding that BCGF-treated cultures have a high fusion efficiency, in no case could we detect the presence of specific antibodies.

5. DISCUSSION

Our findangs raise two important questions. First, given the relatively consistent and strong responses to nonspecific stimuli, why is the response to specific antigenic stimuli so weak and variable? Second, given the number of different factors tested and the generally intense effort that has been expended on in vitro immunization of human lymphocytes over the past 20 years, why have these efforts met with almost universal failure?

Because the nonspecific stimuli do produce good responses, we can conclude that the culture conditions are adequate and probably not limiting. The stimuli that work well with human cells do not always work with cells from other species, but there are some (such as PHA and purified growth factors) that are equally effective on cells from man and mouse. Nor is there any reason to suspect that the human immune system operates in a way that is fundamentally different from that of other species; indeed, when examined closely, the similarities outweigh the differences. In large measure, in vitro immune responses are confined to murine spleen cell populations and in the absence of vigorous prior immunization, good responses are limited to unusual antigens such as sheep erythrocytes (erythrocytes of other species are not nearly as good as those from sheep). Therefore, we might question whether there are any systems that support good primary immune responses; in the mouse it has been well established that allogeneic lymphocytes are among the best agents to induce such primary immune responses, but even then the results are not outstanding. There is no doubt that spleen cells from heavily immunized donors, whether from mice or man, can support a secondary in vitro immune response. Sporadic reports of a "breakthrough" that permits in vitro primary immune responses with human PBL do appear but none have stood the test of time; and as will be noted in the final report, there are in fact good reasons to be suspicious of all such claims. Although there has been little work on in vitro immune responses with human spleen cells or mouse peripheral blood lymphocytes, the available evidence suggests that human spleen cells tend to behave like mouse spleen and mouse PBL like human PBL. Our experience would support this conclusion although we were unable to detect an immune response to DT (a chronic persistent—secondary—antigen for humans) in two experiments (one reported here as experiment 88.8).

In light of these general comments on the limitations of primary in vitro immune responses, we feel it is justified to consider the possibility that the general failure of in vitro primary immune responses is the "correct" result. Accepting negative results is made difficult when there seem to be contradictory examples and there is the ever ready excuse of technical inadequacy. However, recent work in our laboratory on how the humoral immune response functions in vivo sheds some light on this problem.

Extrapolating from the simple observation that antibodies function in a concentration dependent manner, the number of cells producing antibodies can likewise be treated as being present in a concentration that reflects their antibody output. This leads to the imposition of surprisingly stringent limitations on the number of different functional antibodies (or B cells) that an individual can maintain. For example, taking an antibody concentration of 10 ng/ml to the lower threshold for function in clearing an antigen, it requires about 10^3 antibody-producing cells per milliliter to secrete sufficient antibody over a 24 hr period to reach 10 ng/ml. If a minimally protective amount of antibody per ml must be produced in six days, then at time zero there must be at least 10 B cells per ml specific for the antigen in question. To a first approximation every species, from man to tadpoles and elephants, each has close to 10^7 B cells per ml averaged over the total body fluid—effects due to local concentrations of cells in lymph nodes and spleen are thus ignored. Consequently, an individual can recognize in a functional way no more than 10^6 different antigens (assuming each available B cell is potentially inducible). Of course the number of different antibody combining sites generated by somatic mutation is in vast excess of 10^6, and each sample of 10^7 B

cells will have a different set of somatic mutants, but these different antibodies will behave equivalently if the number of potential epitopes on an antigen is large, therefore allowing many different antibodies to find one or another epitope on the antigen—this we refer to as the equivalence principle. One further consequence of this limited antibody repertoire is that antigenic stimulation must cause B cells to proliferate until reaching 10^3 effector cells so that as the steady state epitopic load increases, the amount of space among the 10^7 B cells per ml taken up by proliferating B cells engaged by antigen causes significant numbers of resting B cells to be displaced and thereby compromising the ability of the individual to respond to a new antigen—this we refer to as the "washout" effect.

How do these observations and calculations relate the failure of PBL in vitro immune responses? If we next take into consideration the physiology of lymphoid organs, it is well established that in vivo immune responses occur in compact lymphoid organs, not in blood. Thus, the active population of antigen-induced B cells resides primarily in lymphoid organs and as these cells proliferate they tend to push out quiescent B cells which then circulate in blood until their concentration is reduced back to the overall level of 10^7 per ml. In this case B cells from lymphoid organs would tend to be enriched for those specificities currently in demand whereas in blood there would be a deficit of these cells and an excess of quiescent B cells. However, the quiescent B cells can be divided into two categories, those which are functional and fail to respond due to a lack antigen, and those that are nonfunctional; it is the latter that predominate (the argument as to why this is the case have been published, Langman and Cohn, *Molecular Immunology 24*, 675–697, 1987). Our best estimates at present are that there is as much as a ten-fold dilution of inducible nascent B cells in blood. Under ideal condition we might expect a typical antigen with ten recognizable epitopes to react with 1 in 10^6 to 1 in 10^5 B cells if these cells were homogeneously mixed, but if nonhomogeneity prevails, the actual numbers of blood would be as low as 1 in 10^7. This would make the poor response of PBL to specific antigens predictable, and likewise the good responses found in spleen when animals have been specifically immunized.

If our predictions are correct, it would mean that primary in vitro immune responses will be nearly impossible with present technologies. Consequently, if the goal of obtaining therapeutic quantities of pathogen-specific antibodies were to be realized within the framework of present-day technology, we would suggest that monoclonal antibodies be raised in mice by standard techniques. Then, by straightforward—though tedious—genetic engineering the variable regions of the desired murine monoclonals could be cloned and inserted into appropriate vectors containing the human constant region genes segments. In this way antibodies of the desired specificity *and* isotype could be produced using known technologies.

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