The EMBO Journal vol.5 no.8 pp.1783–1790, 1986

Inhibition of N-linked oligosaccharide trimming mannosidases blocks human B cell development

Abraham Tulp, Marja Barnhoorn, Ernst Bause[1] and Hidde Ploegh

The Netherlands Cancer Institute, (Antoni van Leeuwenhoek Huis), 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands, and [1]Institute for Biochemistry, University of Cologne, FRG

Communicated by H.Ploegh

Deoxymannojirimycin (dMM) or swainsonine (SW), which block conversion of high-mannose to complex-type N-linked glycans, strongly inhibited the production of immunoglobulin (Ig) when added to cultures of human lymphocytes together with the polyclonal B cell activators pokeweed mitogen (PWM) and *Staphylococcus aureus* (SAC). To obtain the inhibitory effect, inhibitor had to be present during the first 36 h of culture. Addition at later timepoints was less effective and showed that neither inhibitor interfered with rate of production or secretion of Ig as such. Viability and proliferation of the lymphocytes, as defined by cell number and rate of DNA synthesis, were not influenced by the presence of dMM or SW, and no changes in the relative number of helper ($T4^+$) or suppressor ($T8^+$) cells were observed. Thus, for normal differentiation of human B lymphocytes into Ig secreting (plasma) cells in response to PWM and SAC, conversion of high-mannose to complex N-linked glycans is essential.

Key words: mannose/oligosaccharide processing/B cell/immunoglobulins/inhibition

Introduction

Proper functioning of the immune system is thought to rely in part on the recognition of carbohydrates by appropriate receptors on lymphoid cells. Homing behaviour of lymphocytes (Gallatin *et al.*, 1983), interaction of helper cells with their target (Muchmore *et al.*, 1980, 1984), NK cell-mediated cytotoxicity (Kiyohara *et al.*, 1985), suppression by T lymphocytes (Tosato *et al.*, 1983; Koszinowski and Kramer, 1981) and the mixed lymphocyte reaction (Cowing and Chapdelaine, 1983) can be modified by treatment with enzymes specific for carbohydrate moieties (O'Brien and Parker, 1976), or by addition of simple sugars. The inhibitor of N-linked glycosylation, tunicamycin (TM), although highly toxic, has also been used to demonstrate the importance of glycosylation in immune reactions (Hart, 1982; Powell *et al.*, 1985).

If protein-bound glycans are in some way involved in bringing about physiological interactions among lymphocytes, then their modification should lead to an altered response. We and others have recently described substances that interfere with N-linked oligosaccharides processing and thus modify N-linked glycan structure (for a review, see Fuhrmann *et al.*, 1985). In this report we show that two such inhibitors, 1-deoxymannojirimycin (dMM; Fuhrmann *et al.*, 1984) and swainsonine (SW; Elbein *et al.*, 1981) interfere with differentiation of human B lymphocytes to immunoglobulin (Ig) secreting plasma cells in response to pokeweed mitogen (PWM) and *Staphylococcus aureus* (SAC) although neither dMM nor SW inhibits Ig secretion *per se*. These observations show that the conversion of high-mannose to complex-type N-linked glycans, a process inhibited by dMM and SW, is a requirement for proper B cell maturation in the PWM–SAC driven system.

Results

Stimulation of human mononuclear cells (MNC)

Human peripheral blood lymphocytes cultured for 5 days in the presence of PWM and SAC produce large quantities of IgG, IgM, and lesser amounts of IgA. For most donors, the combination of PWM and SAC acts synergistically. The synergy between PWM and SAC has been interpreted as evidence in favour of a two-signal model for B cell activation (Saiki and Ralph, 1981; Falkoff *et al.*, 1982). We established optimal conditions of PWM–SAC stimulation (Scholten *et al.*, 1986) leading to maximal synergy. Dependency of the experimental system on intercellular interactions, either direct or by means of soluble factors, was therefore likely to be maximal.

dMM and SW inhibit Ig production, but are not cytotoxic

Whereas no significantly inhibitory effects of dMM and SW on secretion of a number of glycoproteins have been demonstrated (Fuhrmann *et al.*, 1984), both dMM and SW treatment resulted in reduced amounts of Ig recovered from the supernates of PWM–SAC stimulated cultures. For the 20 donors tested, IgM production was inhibited 87% on average by dMM while IgG production was inhibited about 62%. To exclude the possibility that prolonged culture of MNC in the presence of dMM or SW would cause cell death, viability, cell number and rates of DNA synthesis were measured on consecutive days of culture (Figure 1) in the absence and presence of dMM and SW. It is clear that neither inhibitor negatively affects these parameters. Therefore, dMM and SW do not inhibit cellular proliferation as defined by these criteria. Furthermore, when cultures were stimulated with SAC alone, an agent which is mitogenic exclusively for B cells (Schuurman *et al.*, 1980), inclusion of dMM throughout the culture period did not affect DNA synthesis (not shown), indicating that B cell proliferation is normal in the presence of dMM. Moreover, when cultures were incubated with PWM alone, inclusion of dMM throughout the culture even stimulated DNA synthesis about 2.5-fold (data not shown).

dMM and SW affect N-linked glycan structure

The action of both dMM and SW has been characterized previously: dMM leads to retention of high-mannose configuration of N-linked glycans, whereas SW treatment results in hybrid type N-linked oligosaccharides (Gross *et al.*, 1983; Fuhrmann *et al.*, 1985). Enzymatic digestions of Ig with endoglycosidase H and NANase were used to verify inhibition by dMM and SW (data not shown). As non-Ig controls, we routinely analyzed HLA-A,B antigens to afford a diagnosis of N-linked glycan type in cultures where Ig synthesis was strongly inhibited.

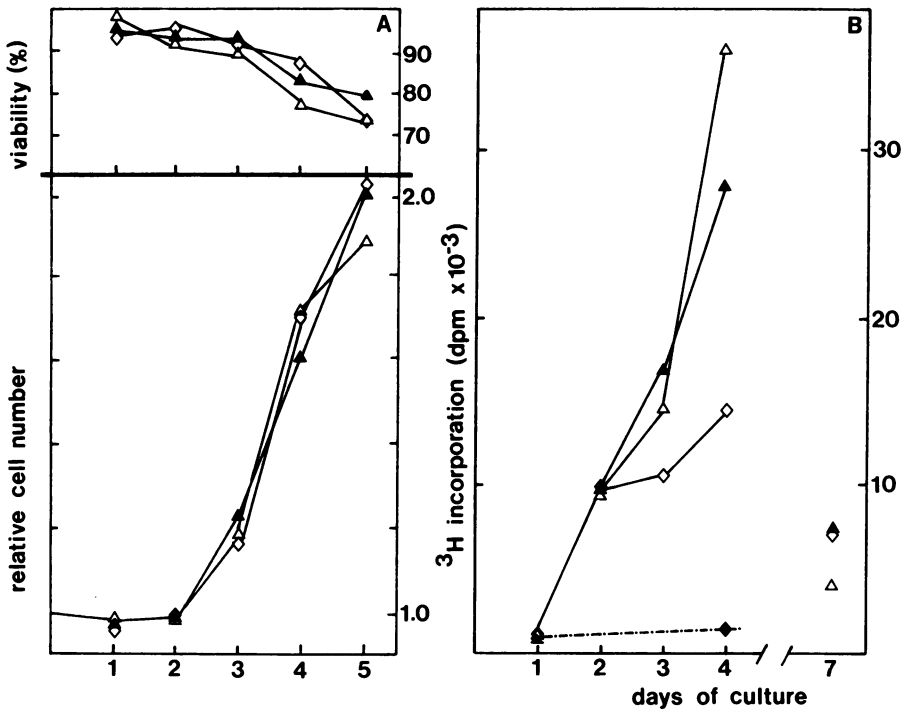

Fig. 1. dMM and SW do not interfere with cell number, cell viability or rate of DNA synthesis. Cultures stimulated with optimal doses of PWM−SAC were maintained in the absence of inhibitor (◇), in the presence of 1 mM dMM (△) or in the presence of 3 μg SW per ml (▲). Viability and cell number were determined after staining of cells with Trypan blue. The fraction of viable cells and cell number were normalized to the start of the culture and are shown in **panel A**. Rates of DNA synthesis were measured as described in Materials and methods and are shown in **panel B**. The dotted line **(panel B)** represents a culture without added PWM−SAC.

Analysis of HLA-A,B antigens, which carry only a single triantennary N-linked glycan, isolated from dMM, SW and control cultures by isoelectric focusing revealed that those from control cultures on average carry two sialic acid moieties, dMM-treated cultures none (Figure 2), and SW-treated cultures only one sialic acid per N-linked glycan (data not shown; Péyrieras *et al.*, 1983). We conclude that in the presence of SW, terminal modifications can take place, resulting in the addition of a single sialic acid per N-linked glycan and acquisition of EndoH resistance. Combining these data, we conclude that both dMM and SW cause changes in N-linked glycan structure consistent with their inhibitory action on trimming mannosidases I and II, respectively (Fuhrmann *et al.*, 1984).

Ig does not accumulate intracellularly in dMM and SW treated cells

The drastically reduced production of Ig in dMM and SW treated cultures could be explained by failure of the cells to properly transport Ig from the site of synthesis to the point of secretion. Therefore lysates of radiolabeled cells were examined for the presence of intracellular μ and γ chains, as identified by immunoprecipitation. Quantitation of the Ig heavy chains recovered in these experiments (included in Figure 6) showed that the amount of intracellular Ig present on day 5 in dMM and SW treated cells is very low and cannot account for the decreased amounts of Ig recovered from the cultured supernates. It should be noted that in PWM−SAC stimulated cultures, significant Ig synthesis becomes detectable only at day 3 or later, but that the inclusion of dMM or SW is required at day 0 or 1 in order to observe the full inhibitory effect on Ig synthesis measured at day 5.

Rate of Ig secretion is not inhibited by dMM

A pulse-chase experiment was carried out to permit a more accurate comparison of the kinetics of Ig secretion in the presence and absence of dMM. Figure 3 shows that secretion of Ig by cells cultured with PWM−SAC for 5 days and thereafter pulse-chased in the presence of dMM, is not inhibited. dMM-Treated cells even secrete Ig at a slightly higher level, when compared with controls. At no time was there any indication that Ig derived from dMM-treated cultures might be more susceptible to degradation than that obtained from control cultures. This observation can explain why dMM, when added to established PWM−SAC stimulated cultures, leads to an increased amount of Ig recovered from the culture supernates (Figure 3 and see below).

dMM and SW do not indiscriminately inhibit glycoprotein expression

To establish that in PWM−SAC stimulated cells dMM and SW are not indiscriminately inhibitory to glycoprotein synthesis we isolated Class I MHC (HLA-A,B) antigens, which are known to be expressed on all nucleated cells. Under conditions where strong inhibition of Ig synthesis is observed, no reduction in the amount of HLA-A,B antigens is seen. Figure 4 shows this result for dMM. Quantitation of HLA-A,B antigens by excision of the labeled material from the gel followed by liquid spectrometry revealed no difference in the amount recovered from dMM-treated and control cultures (not shown). Similar results (not shown) were obtained for SW.

The action of dMM and SW is dependent on time of addition and concentration of inhibitor

When dMM or SW are added at the start of a culture stimulated with SAC and PWM, strong inhibition of Ig production is observed on day 5. Addition of dMM or SW on day 1 likewise produces a strong inhibitory effect, regularly surpassing in magnitude that seen when inhibitors are present from the outset. Ad-

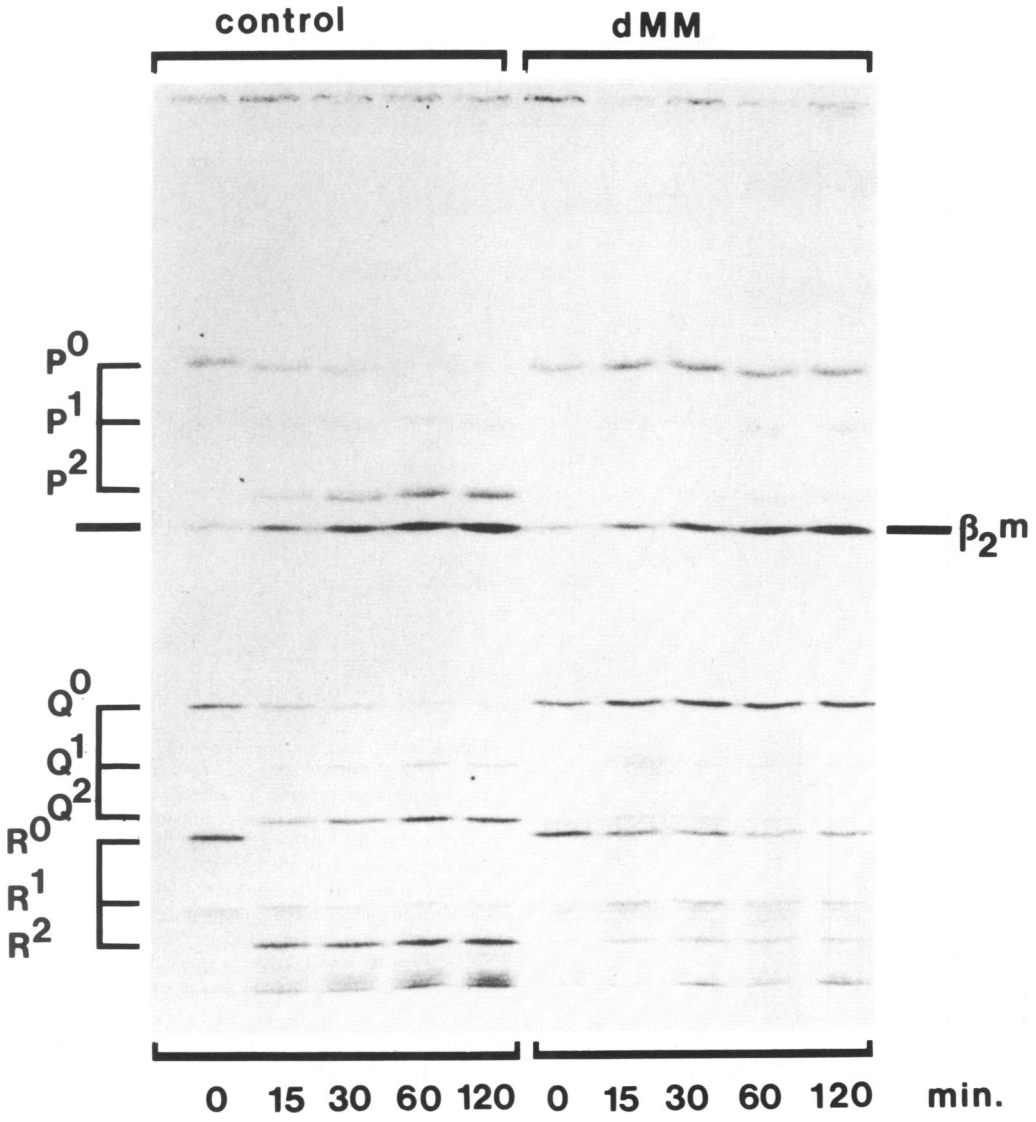

Fig. 2. HLA Class I antigens are not terminally glycosylated in the presence of dMM. Cells stimulated for 5 days with SAC and PWM were pulse labeled in the presence of 1 mM dMM and chased for the times indicated in the figure. HLA Class I antigens were immunoprecipitated with W6/32 and analyzed by IEF. HLA Class I heavy chains are indicated with the letters P, Q and R. The superscripts indicate the number of sialic acid residues present on each heavy chain. Note the shift towards 2-sialic acid-containing forms for control, but not dMM-treated cultures. The position of β_2-microglobulin (β_2m) is indicated. Cathode is at the top.

dition of dMM or SW at later timepoints leads to a progressively weaker inhibition ultimately resulting in a stimulation of Ig production when dMM is added on day 5 (Figure 5). Corresponding quantitative determinations are depicted in Figure 6 for the effect of delayed addition of dMM and SW. It should be emphasized that addition of inhibitor is required at a timepoint preceding significant Ig synthesis, which is on day 3 or later, in PWM−SAC stimulated cultures (Saiki and Ralph, 1981; Scholten *et al.*, 1986).

When the inhibitor is removed from the culture by washing the cells and resuspending them in medium containing PWM and SAC, the inhibition caused by dMM and SW is reversible. The peak of Ig production is shifted to a later timepoint, proportional to the time at which inhibitor was removed (data not shown). Addition of increasing amounts of dMM or SW at the start of the culture shows that near-maximal inhibition is obtained at concentrations of 1 mM (dMM) and 0.5 μg/ml (SW) (Figure 7). These concentrations are similar to those required for full inhibition of N-linked glycan processing (Fuhrmann *et al.*, 1984).

In contrast, when the same dose-response curve is measured for cells cultured with PWM−SAC for 5 days in the absence of dMM or SW, no inhibition is obtained for μ and γ chains recovered from culture supernates even at the highest inhibitor concentrations tested (Figure 8). There is even a pronounced stimulatory effect of the higher dMM concentrations on the production of μ and γ chains, while for SW the level of Ig production is hardly influenced.

Lack of reversibility of the action of dMM by simple sugars

To investigate whether the inhibitory action of dMM might be explained by direct occupation by the inhibitor of a binding site on a lectin-like molecule rather than by inhibitory action on N-linked oligosaccharide trimming, different sugars were added to the culture as competitors. The inhibitory action of dMM could not be relieved by D-mannose (highest concentration tested: 30 mM), L-rhamnose (highest concentration tested: 30 mM) or α-methylmannoside (highest concentration tested: 30 mM) when

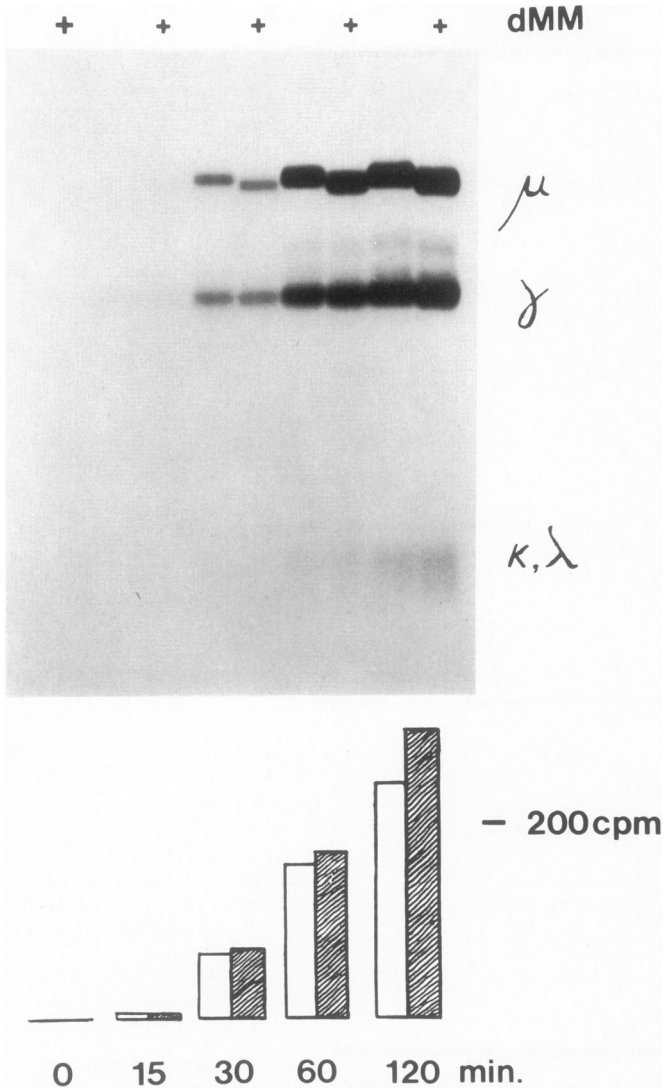

Fig. 3. Pulse-chase analysis of Ig secretion in the presence and absence of dMM. Cells were stimulated with PWM–SAC and labeled with radioactive methionine on day 5 for 10 min in the presence (+) or absence of 1 mM dMM. Afer 5-fold dilution of the cell suspension with methionine-containing medium (chase medium), samples were taken at 0, 15, 30, 60 and 120 min as indicated in the figure. Ig was isolated from the culture supernates and analyzed by SDS–PAGE (upper part). An aliquot of the isolated Ig was quantitated directly by liquid spectrometry (lower part). Hatched bars: dMM-treated cells. Open bars: control cultures.

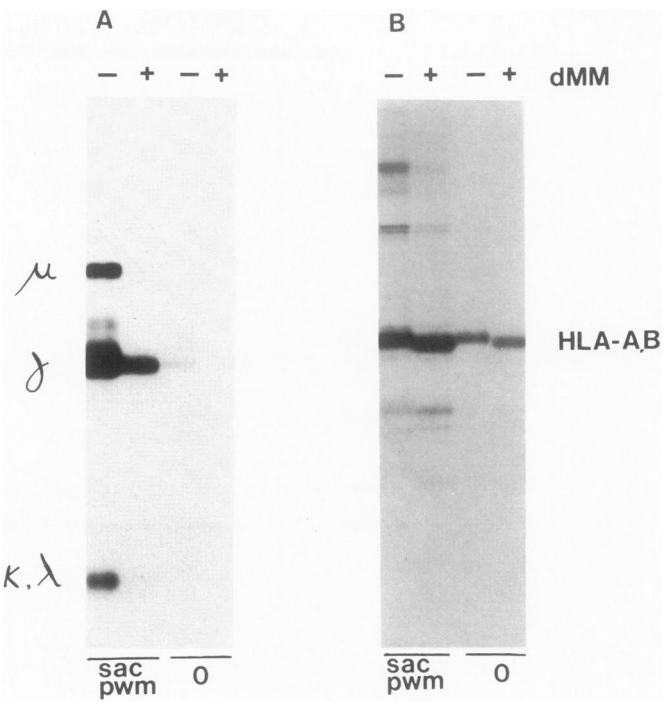

Fig. 4. dMM does not interfere with the synthesis of HLA-A,B antigens. Cells were stimulated with PWM–SAC or medium alone (0) and cultured for 5 days in the presence or absence of 1 mM dMM, after which cells were labeled under the same conditions as they were cultured. Ig was isolated from the culture supernates **(panel A)**, and HLA-A,B antigens from lysates prepared from the labeled cells as described in Materials and methods **(panel B)**. Quantitation of HLA-A,B heavy chains derived from dMM-treated and control cultures by excision of the labeled band and liquid spectrometry revealed no difference in amount. Note the difference in mol. wt between HLA-A,B antigens isolated from control and dMM-treated cultures, due to the different glycan structures.

administered concurrently with dMM. The highest concentration of α-methylmannoside used was slightly inhibitory on Ig production when tested in the absence of dMM, but because of the magnitude of the effect (no more than 20% inhibition), and the concentration required to achieve it (30 mM), this phenomenon was not investigated further.

Analysis of cell populations from dMM-treated and control cultures by fluorescence-activated cell sorting (FACS)

We examined whether major shifts in the composition of the cultures in terms of numbers of helper and suppressor cells had occurred as a consequence of dMM treatment by carrying out immunofluorescent staining with suitable monoclonal antibodies and FACS analysis. Table I shows the results of these experiments. We detected neither changes for the entire T cell fraction as defined by the T3 antigen, nor for the fraction of $T4^+$ (helper) and $T8^+$ (suppressor) cells in cultures maintained in the presence and absence of dMM. Thus the inhibition of Ig production caused by dMM is unlikely to be the consequence of an increased proliferation of T suppressor cells, or a lack of proliferation of T helper cells. There is a drop in HLA Class II antigen-positive cells, both in the presence and absence of dMM as detected by the monoclonal antibody SG 520. The fact that the population of SG 520 (Class II)$^+$ cells decreases sizeably in both control and dMM-treated cultures (Table I) must mean that a large fraction of the reduction in cell viability (Figure 1) is to be found in this population presumably because not every B cell is driven to proliferation in the system. The results shown in Table I also reveal that dMM does not obviously interfere with the surface expression of the T3, T4, T8, Class II and Class I markers, as was previously noted for viral membrane glycoproteins and Class I antigens (Burke *et al.*, 1984).

Discussion

dMM and SW have been described as inhibitors of N-linked glycan processing (Fuhrmann *et al.*, 1985). The action of dMM results in full retention of high-mannose oligosaccharide [structure: $(GlcNAc)_2(Man)_{7-9}$], whereas SW produces oligosaccharide with a $(GlcNAc)_3(Man)_5$ core, onto which further terminal sugars may be added (Gross *et al.*, 1983). In general, the amount of sialic acid that can be incorporated into N-linked glycans will be strongly reduced by treatment with dMM or SW.

dMM and SW have now been tested in a large number of systems for their effects on glycoprotein secretion and on virus assembly, but without any striking inhibitory effects (Burke *et*

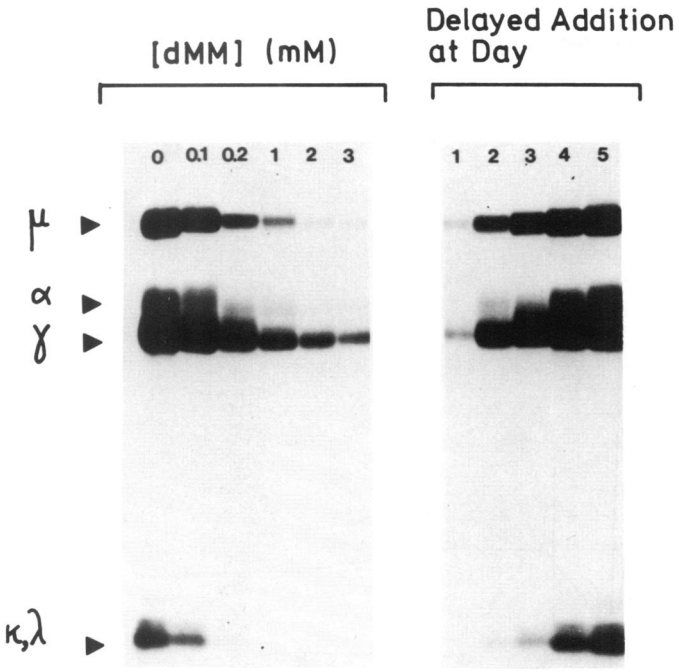

Fig. 5. SDS−PAGE of Ig produced by cultures treated with dMM. Cells stimulated with PWM−SAC were cultured in the presence of increasing concentrations of dMM (left half of autoradiogram) as indicated. Alternatively, cells stimulated with PWM−SAC were cultured in the presence of 1 mM dMM added at day 1, 2, 3, 4 or 5 of culture (delayed addition; right half of autoradiogram). The quantitation shown in Figures 6−8 were performed on electropherograms of the type shown here.

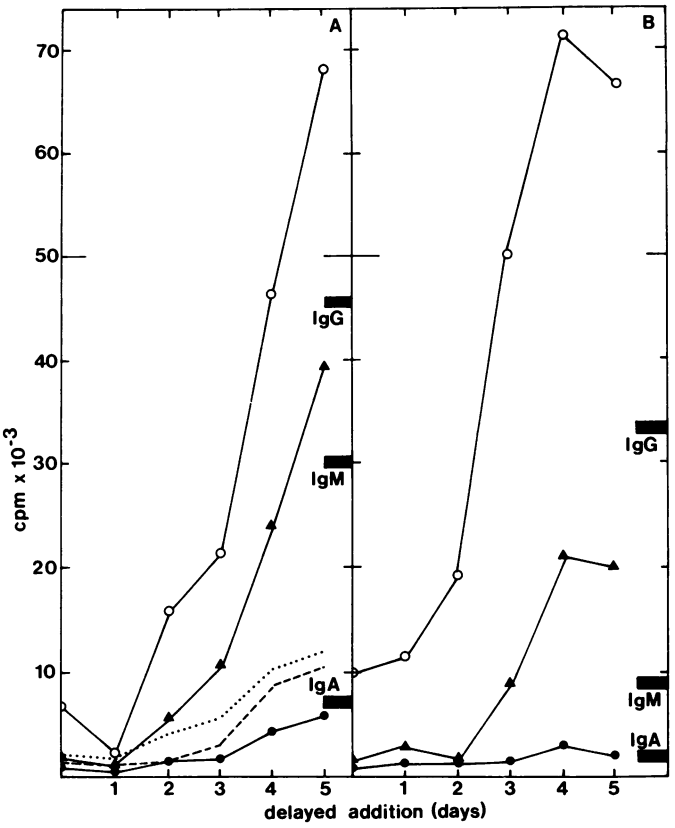

Fig. 6. The magnitude of the inhibitory effect of dMM and SW on Ig production is determined by the time of addition. Cultures were started in the presence of optimal doses of PWM and SAC. Inhibitors (1 mM dMM, **panel A**, or 3 μg SW per ml, **panel B**) were added on day 0, 1, 2, 3, 4 or 5 and left in culture for the remaining period (delayed addition). Different donors were used for **panels A** and **B**, explaining the difference in levels of Ig secreted by non-treated cultures. Cultures were labeled on day 5 with 35S-methionine in the presence of inhibitor, and synthesis of μ (▲), γ (○) and α (●) chains was quantitated by cutting out bands from dried SDS−PAGE gels as described in Materials and methods. Values for control cultures (no inhibitor present during culture and labeling) are indicated by horizontal solid bars. In the case of dMM (**panel A**), radioactivity for μ (----) and γ (. . . . . .) chains remaining associated with the cells (intracellular Ig) after labeling was also determined.

al., 1984). The apparent lack of toxicity of dMM and SW in tissue culture suggests that the integrity of this part of the oligosaccharide trimming pathway is not crucial for survival of the individual cell at least *in vitro*. However, cellular interactions have been shown to depend at least in part on carbohydrate structures (Turner and Burger, 1973; Lennarz, 1985), so that a carefully maintained balance and specificity in conversion of high-mannose to complex-type N-linked glycans may be essential. We have therefore studied the effects of dMM and SW in a system that is strictly dependent on such cellular interactions, the differentiation of human MNC driven by PWM−SAC. In this easily accessible model, monocytes, T cells and B cells interact, in part directly, in part by means of factors they produce to stimulate cell division and differentiation. Ultimately, B cells develop into Ig-secreting plasma cells after 5−6 days of culture.

The results obtained with dMM and SW clearly show that N-linked glycan processing is a necessary requirement for high levels of Ig production in the PWM−SAC driven system. Having ruled out trivial explanations such as effects of dMM and SW on cell number, viability and proliferation, we conclude that dMM and SW are apparently capable of uncoupling cell division from differentiation in this sytem. It is particularly striking that maximal inhibition of Ig production requires addition of dMM at a timepoint (day 1) when no DNA synthesis or Ig secretion is apparent. The results presented in Figure 1, in conjunction with our observation that stimulation with SAC alone, an agent specifically mitogenic for B lymphocytes (Forsgen *et al.*, 1976; Schuurman *et al.*, 1980), gave similar values of [^{3}H]thymidine incorporation for dMM-treated and control cultures, suggest that B cell proliferation was normal in the presence of dMM. Furthermore, inclusion of PWM alone in the culture stimulated DNA synthesis about 2.5-fold in the presence of dMM indicating that the obligatory first step of PWM stimulation, i.e. presentation by monocytes to T cells (Stevenson *et al.*, 1983), is not affected by the trimming inhibitor. In combination with the results obtained by FACS analysis, we conclude that there are no major differences in cellular composition and proliferation as a consequence of dMM treatment.

We attribute the observed effects to the inhibitory action of dMM and SW on N-linked glycan processing. First, dMM and SW — although equally inhibitory on Ig production — are structurally different, but share the property of inhibiting trimming mannosidases. Second, D-mannose, L-rhamnose or α-methyl-mannoside were incapable of relieving the inhibitory effect of dMM and SW, suggesting that it is unlikely that dMM or SW themselves act as ligands for a mannose-specific receptor that might be involved in B cell differentiation. Third, the concentrations at which dMM and SW block Ig production are similar to those needed to achieve inhibition of trimming mannosidases.

The effects of dMM and SW should be interpreted in the framework of other reports implicating carbohydrates in lymphocyte function. The ability of a wide range of lectins to stimulate proliferation of lymphocytes or intervene in interactions amongst lymphocytes, hardly needs further comment, and shows that car-

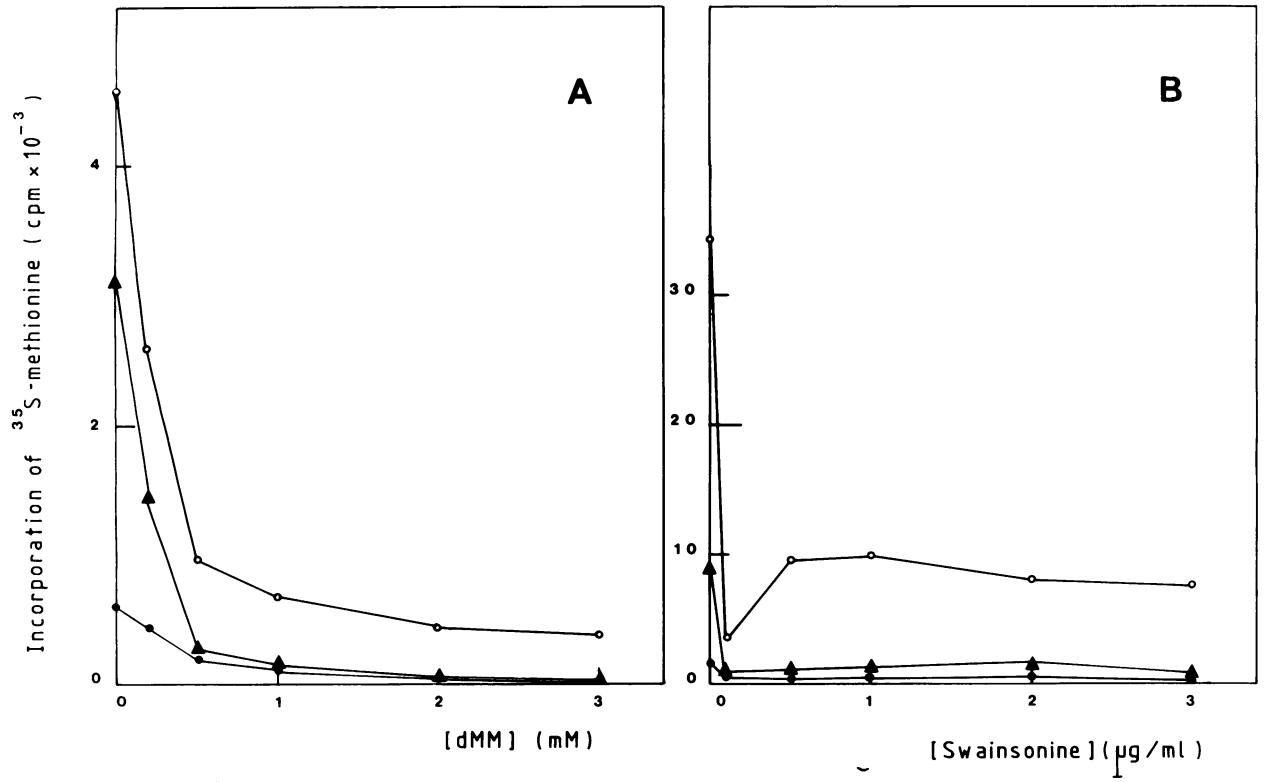

Fig. 7. Dose-response curves for inhibition of Ig production by dMM and SW. PWM–SAC stimulated cultures were maintained in the presence of increasing amounts of the inhibitors dMM or SW and were labeled on day 5 of culture. Ig was isolated from the culture supernates as described in Materials and methods. After SDS–PAGE, bands corresponding to μ (▲), γ (○) and α (●) chains were cut out and quantitated by liquid spectrometry. Radioactivity recovered for each species of heavy chain is plotted as a function of inhibitor concentration. **Panel A:** dMM; **panel B:** SW.

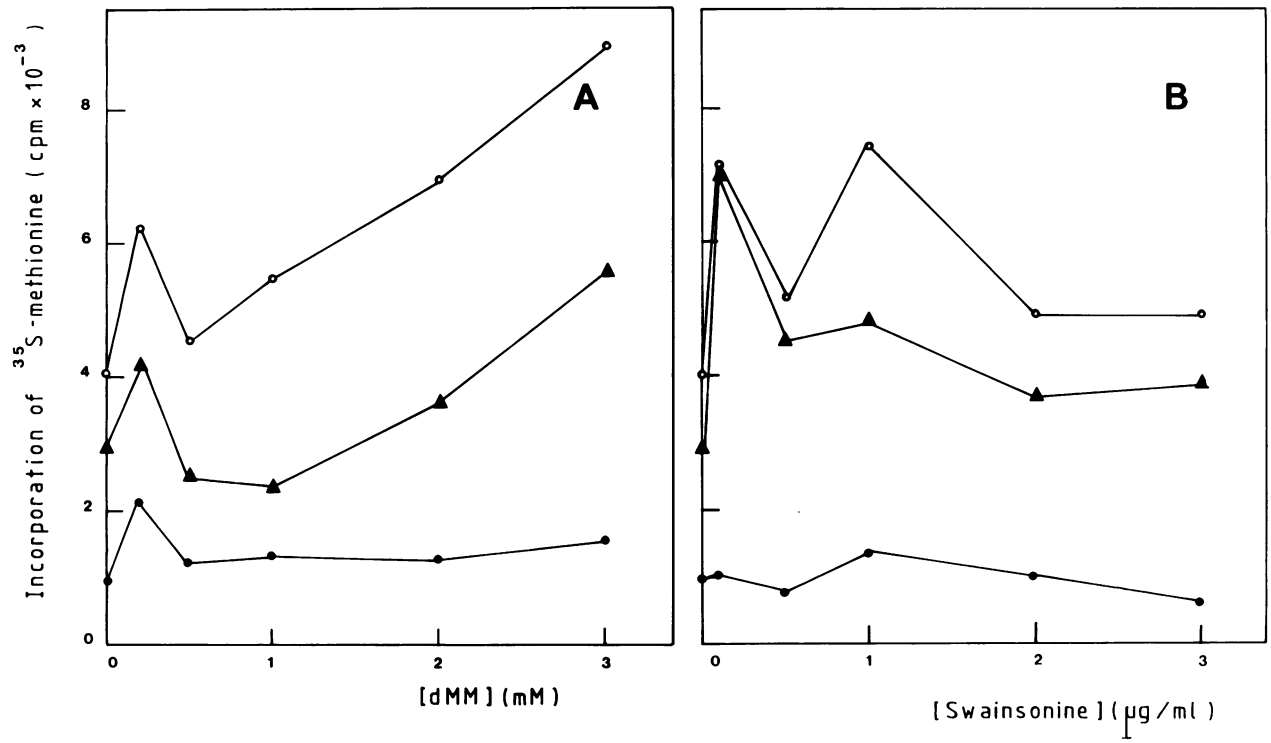

Fig. 8. Effect of dMM and SW on secretion of Ig as a function of inhibitor concentration. Labeling of cells and quantitation of μ (▲), γ (○) and α (●) chains as described in Figure 6. Instead of adding inhibitor to the culture from the outset, cells were stimulated by PWM–SAC and allowed to develop in the absence of inhibitor for 5 days, after which Ig production was measured as a function of inhibitor concentration. Inhibitor was added 1 h prior to the addition of label, and was continuously present during labeling. **Panel A:** dMM; **panel B:** SW.

Table I. FACS analysis of MNC prior to and after 5 days of culture in the absence and presence of dMM in PWM−SAC stimulated cultures

	Treatment		
	Control Day 0	Control Day 5	dMM Day 5
Antibody			
W6/32	0.96	0.85	0.87
SG520	0.33	0.16	0.16
T3	0.66	0.71	0.73
T4	n.d.	0.46	0.46
T8	n.d.	0.26	0.27

Data are presented as the fraction of positive cells.

bohydrate-specific interactions are capable of promoting a biological response (Sharon, 1984).

Infection with Epstein−Barr virus (EBV) has been reported to lead to the generation of T suppressor cells that are capable of suppressing a PWM driven system as measured by Ig production (Tosato and Blaese, 1985; Tosato *et al.*, 1979, 1983). The suppressive effects of these T cells could be overcome by the addition of D-mannose in mM concentrations, suggesting that a mannose recognizing system is involved in this type of suppression (Tosato *et al.*, 1983). Further evidence in favour of the involvement of a mannose-specific recognition system stems from the observation that the disaccharide α-1,6-mannosylmannoside exerts a strongly suppressive effect on antigen-specific T cell proliferation in cultures of human MNC (Muchmore *et al.*, 1984). A mannose-specific receptor has in fact been identified on cells of the reticuloendothelial system including macrophages (Stahl *et al.*, 1978), but it is unclear whether this receptor is involved in the immunosuppressive phenoma alluded to above.

Can the results reported here and the data available from the literature be integrated into a single coherent picture? The structures of the N-linked glycans produced by the action of dMM and SW have in common an unmodified antenna with an α-1,6-mannosylmannoside linkage, absent from complex-type glycans. We might therefore postulate, in keeping with the results reported for the isolated disaccharide α-1,6-mannosylmannoside (Muchmore *et al.*, 1984) that this moiety is responsible for the suppressive effect ultimately observed. Cells expressing this structure, either having produced it biosynthetically or having acquired it by absorption from the surrounding medium, may be a superior target for suppressor cells. However, we have observed that elimination of $T8^+$ cells does not abolish inhibition by dMM of Ig production after PWM−SAC stimulation (data not shown), arguing against this possibility. Alternatively, cells providing positive stimulatory signals may become incapacitated by the presence of aberrant N-linked glycans, even if present on these very same cells. Such a phenomenon might be considered analogous to the inability of the hepatic asialoglycoprotein receptor to participate in receptor-mediated endocytosis in cells treated with neuraminidase (Stockert *et al.*, 1977), which results in the exposure of galactose moieties recognized by neighbouring receptors on the same cell. The $T4^+$ (helper) cell would then be an obvious candidate for the target of action of dMM.

It is conceivable that the production or activity of lymphokines is affected by dMM or SW. However, it should be noted that recombinant non-glycosylated interleukins retain biological activity as was shown for IL-1 (Lomedico *et al.*, 1984), BSF-1 (Noma *et al.*, 1986) and IL-2 (Rosenberg *et al.*, 1984) and that the mannosidase inhibitors do not inhibit secretion of glycoproteins examined to date (Fuhrmann *et al.*, 1985).

The present results demonstrate for the first time that a relatively complicated system involving cellular interactions is dependent on proper N-linked glycan maturation for differentiation, but not cell division, to occur. The use of the non-toxic inhibitors dMM and SW is clearly an advantage in such complicated systems. They permit conclusions on the significance of types of glycans, as opposed to their complete absence or presence. Other treatments that allow modification of N-linked glycans are often incompatible with viability of cells, as is the case for most chemical modifications, or do not allow a distinction between N- and O-linked sugars or even glycolipids, as exemplified by neuraminidase treatment. So far, it has been impossible to eliminate N-linked glycans from living cells by treatment with substances other than TM and 1-deoxyglucose, which, as already mentioned, are poorly tolerated by most tissue culture cells. It would be interesting to probe other systems used to study development with inhibitors such as dMM or SW. One obvious choice would be to examine embryonic development, where carbohydrates have been shown to undergo structural changes with time (Edelman, 1983). In particular, molecules mediating cellular adhesion deserve close attention, and may shed further light on the functional significance of N-linked glycans in differentiation and development.

Materials and methods

Cells

20−40 ml heparinized blood were obtained by venipuncture from healthy volunteers. MNC were prepared after 1:1 dilution by flotation on Ficoll-Hypaque (Boyum, 1977). Interface cells were washed three times and resuspended in RPMI 1640.

Cell culture

One million MNC were cultured in 1 ml of RPMI supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin−streptomycin (at 100 units/ml each) in a humidifed CO_2 incubator at 37°C using Falcon (Becton-Dickinson, Oxnard, CA) 24-well tissue culture plates. Mitogens and dMM or SW were added at concentrations as indicated in the text and the figures.

Preparation of SAC

SAC Cowan I was prepared as described by Kessler (1975), but with the inclusion of a second formaldehyde fixation (Schuurman *et al.*, 1980) to render the SAC entirely B-cell specific. Stocks were stored at −70°C as a 10% (v/v) suspension. Prior to addition to cell cultures, SAC were washed three times by resuspension in RPMI 1640 medium followed by centrifugation. The final suspension was adjusted to 10% (v/v), and dilutions were prepared from this stock.

Mitogenic stimulation

PWM was obtained from Gibco (Grand Island, NY) and reconstituted as per suppliers instructions. PWM (5 μl) and SAC (0.0005%) were added to pre-warmed culture medium.

Labeling procedures

At the times indicated in Results, samples (10^6 cells) were withdrawn from culture and harvested by centrifugation. Cells were resuspended in RPMI 1640 medium without methionine and supplemented with 10% FCS. 35S-Methionine (1200 Ci/mmol, Amersham, UK) was added to a concentration of 10 μCi/ml, and incubation was continued for 16 h in a humidified CO_2 incubator. Cells were harvested by centrifugation and the supernates were used directly for isolation of Ig. Lysates were prepared from the labeled cells as described (Ploegh *et al.*, 1981) and used immediately for isolation of Ig ('cytoplasmic Ig') or HLA-A,B antigens.

Isolation of Ig

Ig were isolated from the culture supernates by incubation with 50 μl of a 10% SAC suspension prepared as described above. IgG, and a fraction of IgA and IgM, will bind to SAC at this stage. The SAC with adsorbed Ig were washed as described. Ig remaining in the culture supernates after incubation with SAC, largely consisting of IgM and IgA, was recovered by immunoprecipitation with a mixture of rabbit anti-human IgM, anti-human IgG and anti-human IgA sera [Behring Werke, Marburg (FRG) and Central Laboratory of the Blood Trans-

fusion Service, Amsterdam (NL)]. The immunoprecipitation protocol has been described in detail (Ploegh *et al.*, 1981). Since the results obtained by direct precipitation with SAC and those obtained by the second round of precipitation with anti-Ig reagents were similar the experiments described from the first round of precipitation are shown in the figures.

Isolation of HLA-A,B antigens

HLA-A,B antigens were isolated by immunoprecipitation from detergent lysates (Ploegh *et al.*, 1981) using the monoclonal antibody W6/32 (Parham *et al.*, 1979), which recognizes all HLA-A and -B antigens.

Enzyme digestions

Ig or HLA-A,B antigens adsorbed to SAC were digested with EndoH (Boehringer-Mannheim, or Miles) or NANase (Type VIII, Sigma, St. Louis, MO) as described (Burke *et al.*, 1984).

Cell number and viability

Cell counting was performed in a Coulter counter (model ZF, Harpenden, UK). Viability was measured by Trypan blue exclusion. DNA synthesis was measured by pulse-labeling cells for 2 h with 1 μCi tritiated thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA). Cells were chilled, centrifuged and washed with phosphate-buffered saline, and precipitated with 5% (w/v) trichloroacetic acid. After two washes with 5% (w/v) trichloroacetic acid radioactivity was measured by liquid spectrometry.

Gel electrophoretic techniques

SDS–PAGE was carried out as described (Dobberstein *et al.*, 1979). After localization of bands by fluorography, bands corresponding to Ig or HLA-A,B heavy chains were cut out from the dried gels, rehydrated and dissolved in 1 ml of Soluene (Packard Zurich, Switzerland) at 50°C for 6 h. Ten milliliters of Instagel (Packard) supplemented with 50 μl of glacial acetic acid were added, and samples were quantitated by liquid scintillation counting. Isoelectric focusing was carried out as described by Neefjes *et al.* (1986).

Pulse-chase analysis of Ig secretion in the presence and absence of dMM

Cells were cultured in the presence of PWM–SAC, and on day 5 labeled for 10 min in the presence or absence of dMM. After 5-fold dilution of the cell suspension with methionine-containing medium (chase medium), samples were taken at 0, 15, 30, 45, 60 and 120 min as indicated in the figure. Ig was isolated from the culture supernates and analyzed by SDS–PAGE. An aliquot of the isolated Ig was quantitated directly by liquid spectrometry.

FACS analysis

Cells were treated with MAbs W6/32, SG520, OKT3, OKT4 and OKT8, and after washing treated with FITC-labeled goat anti-mouse Ig as described by Reinherz and Schlossman (1980). SG520 recognizes HLA Class II antigens, OKT3, OKT4, and OKT8 define the total human T cell subset, helper-inducer T cells and suppressor cytotoxic T cells, respectively (Reinherz and Schlossman, 1980). Cells were then analyzed using a FACS IV cell sorter (Becton-Dickinson, Oxnard, CA).

Chemicals

α-methylmannoside and L-rhamnose were from Sigma Chemical Co., St. Louis, MO. SW was a gift of Dr Vosbeck. dMM can be obtained commercially from Biosyntech GMbH, Hamburg, FRG.

Acknowledgements

This research was supported in part by project grant NKI 85-9 of the Queen Wilhelmina Foundation (KWF).

References

Boyum,A. (1977) *Lymphology*, **10**, 71–76.
Burke,B., Matlin,K., Bause,E., Legler,G., Peyrieras,N. and Ploegh,H. (1984) *EMBO J.*, **3**, 551–556.
Cowing,C. and Chapdelaine,J.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6000–6004.
Dobberstein,B., Garoff,H. and Warren,G. (1979) *Cell*, **17**, 759–769.
Edelman,G.M. (1983) *Science*, **219**, 450–457.
Elbein,A.D., Solf,R., Dorling,P.R. and Vosbeck,K. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7393–7397.
Falkoff,R.J.M., Zhu,L.P. and Fauci,A.S. (1982) *J. Immunol.*, **129**, 97–102.
Forsgren,A., Svedjelund,A. and Wigzell,H. (1976) *Eur. J. Immunol.*, **6**, 207–213.
Fuhrmann,U., Bause,E., Legler,G. and Ploegh,H. (1984) *Nature*, **307**, 755–758.
Fuhrmann,U., Bause,E. and Ploegh,H.L. (1985) *Biochim. Biophys. Acta*, **825**, 95–110.
Gallatin,W.M., Weissman,I.L. and Butcher,E.C. (1983) *Nature*, **304**, 30–34.
Gross,V., TranThi,T.A., Vosbeck,K. and Heinrich,P.C. (1983) *J. Biol. Chem.*, **258**, 4032–4036.
Hart,G.W. (1982) *J. Biol. Chem.*, **257**, 151–158.
Kessler,S.W. (1975) *J. Immunol.*, **115**, 1617–1623.
Kiyohara,T., Dennis,J.W., Boegman,R.J. and Roder,J.C. (1985) *J. Immunol.*, **135**, 659–664.
Koszinowski,U.H. and Kramer,M. (1981) *Nature*, **289**, 181–184.
Lennarz,W.J. (1985) *Trends Biochem. Sci.*, **10**, 248–251.
Lomedico,P.T., Gubler,U., Hellmann,C.P., Dukovich,M., Giri,J.G., Pan,Y.E., Collier,K., Semionow,R., Chua,A.O. and Mizel,S.B. (1984) *Nature*, **312**, 458–462.
Muchmore,A.V., Decker,J.M. and Blaese,R.M. (1980) *J. Immunol.*, **125**, 1306–1311.
Muchmore,A.V., Decker,J.M., Blaese,R.M. and Nilsson,B. (1984) *J. Exp. Med.*, **160**, 1672–1685.
Neefjes,J.J., Breur-Vriesendorp,B., Van Seventer,G., Ivany,P. and Ploegh,H.L. (1986) *Human Immunol.*, in press.
Noma,Y., Sideras,P., Naito,T., Bergstedt-Lindquist,S., Azuma,C., Severinson, E., Tanabe,T., Kinashi,T., Matsuda,F., Yaoita,Y. and Honjo,T. (1986) *Nature*, **319**, 640–646.
O'Brien,R.L. and Parker,J.W. (1976) *Cell*, **7**, 13–20.
Parham,P., Barnstable,C.J. and Bodmer,W.F. (1979) *J. Immunol.*, **123**, 342–349.
Péyrieras,N.M., Bause,E., Legler,G., Vasilov,R., Claesson,L., Peterson,P. and Ploegh,H.L. (1983) *EMBO J.*, **2**, 823–832.
Ploegh,H.L., Orr,H.T. and Strominger,J.L. (1981) *Cell*, **24**, 287–299.
Powell,L.D., Bause,E., Legler,G., Molyneux,R.J. and Hart,G.W. (1985) *J. Immunol.*, **135**, 714–724.
Reinherz,E.L. and Schlossman,S.F. (1980) *Cell*, **19**, 821–827.
Rosenberg,S.A., Grimm,E.A., McGrogan,M., Doyle,M., Kawasaki,E., Koths,K. and Mark,D.F. (1984) *Science*, **223**, 1412–1415.
Saiki,O. and Ralph,P. (1981) *J. Immunol.*, **127**, 1044–1047.
Scholten,P., Schuurman,R.K.B. and Ploegh,H.L. (1986) *Human Immunol.*, **16**, 1–13.
Schuurman,R.K.B., Gelfand,E.W. and Dosch,H. (1980) *J. Immunol.*, **125**, 820–826.
Sharon,N. (1984) *Trends Biochem. Sci.*, **9**, 198–202.
Stahl,P.D., Rodham,J., Miller,M.J. and Schlesinger,P.H. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1399–1403.
Stevenson,H.C., Miller,P.J., Waxdal,M.J., Haynes,B.F., Thomas,C.A. and Fauci,A.S. (1983) *Immunology*, **49**, 633–640.
Stockert,R.J., Morell,A.G. and Scheinberg,I.H. (1977) *Science*, **197**, 667–668.
Tosato,G. and Blaese,R.M. (1985) *Adv. Immunol.*, **37**, 99–146.
Tosato,G., Magrath,I., Koski,I., Dooley,N. and Blaese,M. (1979) *N. Engl. J. Med.*, **301**, 1133–1137.
Tosato,G., Pike,S.E. and Blaese,R.M. (1983) *J. Exp. Med.*, **158**, 1048–1060.
Turner,R. and Burger,M. (1973) *Nature*, **244**, 509–510.

Received on 24 April 1986