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Monoclonal antibodies and cell surface antigens

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Abstract Antibody chains are encoded in three gene clusters containing genes for the variable and constant regions. V and C genes are separated in the germ line and during differentiation a rearrangement takes place. But even after this rearrangement the V and C coding sequences are not contiguous. A final splicing must take place in committed cells between the transcription of a discontinuous V- and C-region DNA and the expression of a continuous mRNA coding for an antibody chain. Analysis by cell fusion indicates that the splicing is *cis*.

When two antibody-producing cell lines are fused, the resulting hybrids express the two antibodies that characterize the parental lines. Permanent cell lines producing antibody of predefined specificity have now been derived in this way. Spleen cells from hyperimmunized donors are fused with myeloma cells and a proportion of the hybrids that are established synthesize and secrete antibodies directed against the immunogen. The heterogeneous cell population can be cloned and propagated. This is a potent way of producing monospecific antibodies to complex antigens such as cell membranes and transplantation antigens.

Monoclonal xenogeneic antibodies to rat cell-surface membranes have proved very valuable for characterizing and separating rat lymphocyte subpopulations. In more recent experiments, monoclonal xenogeneic antibodies to mouse and human cell-surface antigens have also been produced which permit the characterization of the hitherto undescribed differentiation antigens.

When an animal is injected with cells from another animal of a different species or a different strain, an antibody response is initiated. This response involves the production of circulating antibodies directed against the injected cells. Cells contain a large number of surface antigens, i.e. structures which are recognized as non-self by the immune system of the responding animal.

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Each antigen is recognized by different antibodies directed to different regions (i.e. independent antigenic determinants), but each antigenic region can be recognized by a number of antibodies directed to overlapping determinants, and even the same determinant is usually recognized by several antibody structures. In this way an enormously complex and heterogeneous response is initiated which is the hallmark of the antibody system. According to the clonal selection theory each antibody-producing clone is committed to the production of a single antibody structure which is selected by the antigen for further proliferation (Burnet 1969). The heterogeneity is therefore built up by an enormous complexity of clones of responding cells.

Tumours of immunoglobulin-producing cells either occur spontaneously, like the myelomas in man, or can be induced experimentally. These tumours produce and secrete large amounts of myeloma protein, which is an immunoglobulin with an unknown antibody activity. Myeloma proteins, therefore, are a random collection of antibodies directed against unknown antigens. Myeloma proteins, however, were for a long time the only source of pure Ig preparations and were for that reason an invaluable tool in structural studies.

Antibodies are made up of light and heavy chains. Each of these chains consists of two segments, one referred to as the variable and the other as the constant region (Hilschmann & Craig 1965). The antibody combining site is defined by the amino acid sequence of the variable region which determines the unique diversity in protein structure that is characteristic of the immune system. The realization that V regions were coded by multiple V genes while C regions could be coded by single genes (Milstein 1967) made inescapable the assumption that V and C genes should occur separately in the genome (Dreyer & Bennett 1965). This has now been supported in a more direct way by the difference in restriction fragments of DNA derived from myelomas and embryonal or other tissues. At some stage in differentiation, therefore, rearrangement of DNA involved in antibody synthesis takes place. But it appears that even in myeloma cells, after this rearrangement has taken place, V- and C-region DNA sequences are not contiguous (Tonegawa *et al.* 1976; Rabbitts & Forster 1978; Brack & Tonegawa 1977). On the other hand, sequence analysis of mRNA indicates that the V and C sequences are uninterrupted and that the protein is translated from a single continuous mRNA (Milstein *et al.* 1974). So a final rearrangement or splicing takes places somewhere between the transcription of a discontinuous V- and C- myeloma DNA and the expression of a continuous mRNA.

HYBRID MYELOMA CELLS AND THE SPECIFICITY OF V–C INTEGRATION

We further know that the splicing or rearrangement is *cis*, i.e. that it does not involve the production of a pool of V- and C-region precursor RNA or DNA molecules. This information comes from an experiment done by Cotton & Milstein (1973) in which hybrid cells expressing two different sets of V- and C-region kappa light chains were produced. The C-region difference was ensured by using cells of mouse and rat origin (Fig. 1). The experiment

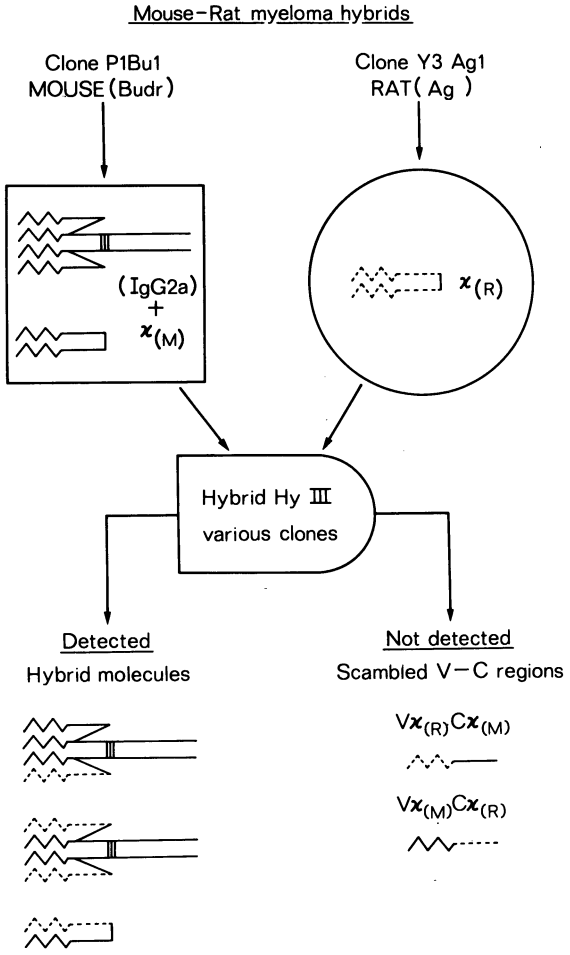

Fig. 1. Codominant *cis* expression of antibody genes in hybrids of myeloma cells. The data are presented in Cotton & Milstein (1973). Diagram reproduced from Milstein & Köhler (1977).

described in Fig. 1 was by necessity between mouse and rat cells because the C regions of kappa light chains of mice have no known genetic markers and therefore no distinction between two different C_χ regions of mice was possible.

Markers for the heavy chains are much more simple because the V_H region is shared by all classes and subclasses of heavy chains. The random expression of different combinations of V_H and C_H on cells synthesizing two different heavy chains can therefore be checked, even within the same strain of mice. Experiments along these lines confirmed the general conclusions drawn from the experiments of Fig. 1 (Köhler & Milstein 1975; Milstein *et al.* 1977; Margulis *et al.* 1977).

This type of observation has been extended more recently to a large number of other examples with other myelomas and antibody-producing cells, including different types and classes of heavy chains. In some experiments efforts have been made to induce trans-integration but with no success. In other words, hybrid cells which express two immunoglobulins, one of the μ class, capable of lysing sheep red blood cells (SRBC), and another, an IgG1 immunoglobulin with no antibody activity, retain the separate expression. Out of 10^5 clones, none was found which expressed the anti-SRBC activity in IgG1 molecules. A similar result was obtained with an anti-TNP (trinitrophenyl)-producing hybrid (Milstein *et al.* 1977, and unpublished experiments).

DERIVATION OF ANTIBODY-PRODUCING LINES BY CELL FUSION

Fusion of two immunoglobulin-producing cells therefore results in the codominant expression of both immunoglobulins. Mixed molecules expressing different combinations of H and L chains are produced by hybrid cells but not by a mixed population of cells, indicating that the mixed molecules are formed intracellularly (Cotton & Milstein 1973; Köhler & Milstein 1975). These experiments suggested the production of hybrids between a myeloma cell producing an immunoglobulin with no known antibody activity but with the ability to grow permanently in culture, and another cell capable of producing a well-defined antibody but with a limited lifespan, as it is an antibody-producing cell of an animal (Köhler & Milstein 1975). Such fusions (Fig. 2) produced permanent hybrid lines capable of permanent growth in tissue culture and as transplantable tumours, which have the capacity to produce the specific antibody expressed by the normal primed parental cell. The first antibodies produced in this way were anti-SRBC and anti-TNP hapten antibodies (Köhler & Milstein 1975, 1976; Milstein & Köhler 1977).

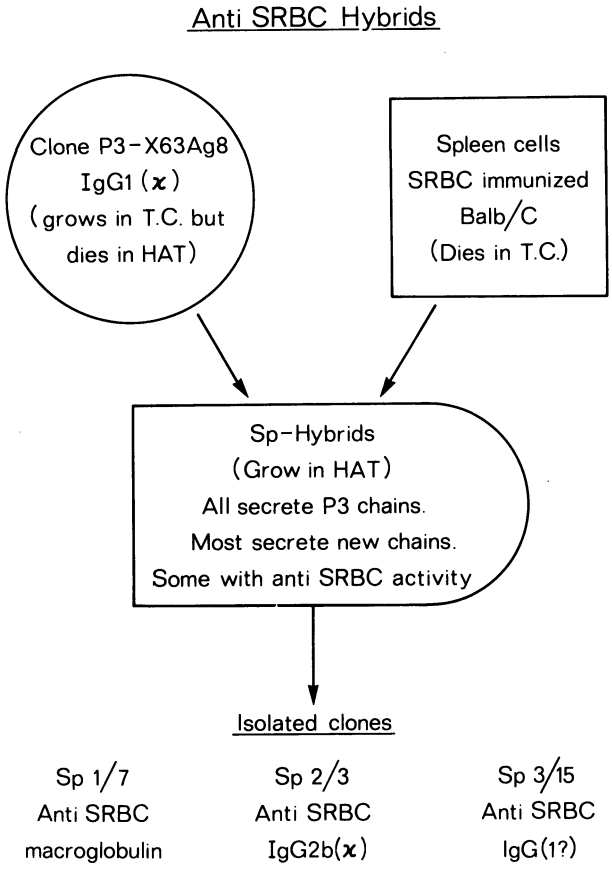

Fig. 2. Fixation of specific antibody production by a transient spleen cell in a permanent tissue culture line.

Technical modifications in the fusion technique, screening and purification of active clones were introduced for the derivation of hybrid myelomas secreting monoclonal antibodies to the cell surface antigens of the major histocompatibility complex of the rat (Galfre *et al.* 1977). Since then a large number of other antibodies have been produced (Table 1), and the results obtained formed the subject of a recent workshop (Melchers *et al.* 1978). The experience accumulated so far tends to indicate that the procedure is quite general and that any antibody an animal can produce can also be prepared as a monoclonal antibody through myeloma hybrid lines.

The major interest of this technique derives from two fundamental points. First, the monoclonal antibody produced by an isolated clone is a well-defined

TABLE 1

Examples of monoclonal antibodies produced (by us and others) using the hybrid-myeloma technique

Antibodies against:
red blood cells (sheep, human)
haptens (TNP, NP)
proteins (γ-globulins)
carbohydrate (bacterial cell wall, Forssman antigen)
enzymes (peroxidase, lysozyme)
viruses (influenza)
major histocompatibility antigens
other cell surface components (differentiation antigens)

chemical and not an undefined heterogeneous mixture which changes with each immunized animal and even with each bleed of the same animal. The permanent cultures are capable of producing an unlimited supply of exactly the same chemical structure. Second, the technique is ideally suited for the preparation of pure antibodies using non-purified antigens.

PURE ANTIBODIES FROM IMPURE ANTIGENS

As pointed out in the introduction, the antibody response of an animal to the challenge of a given antigen is highly complex. This is particularly so when cells from a given species are injected into an animal of a different species. The serum of the immunized animal contains a large number of antibodies directed against a large number of cell surface components. Multiple antibodies are likely to be produced against a single antigen and even against a single antigenic determinant (Fig. 3).

There is a more interesting complication. The immunizing cells are often themselves a complex set of different cell types; the antigenic structures shown in the hypothetical cell of Fig. 3 used as immunogen are therefore somewhat different in different cell types. These antigens, present in some cell types but not in others, are referred to as differentiation antigens. They allow us to recognize and separate one cell type from another. An extreme example of cell heterogeneity which can only be distinguished by the antigenic character of the cell surface are those B cells which differ only in the structure of the (antibody) receptor they synthesize.

The consequence of such complex antigenic stimulation is an even more complex antibody response. The use of such complex antibody mixtures is severely restricted and requires extensive purification. However, each antibody-producing cell is committed to the synthesis of a single antibody.

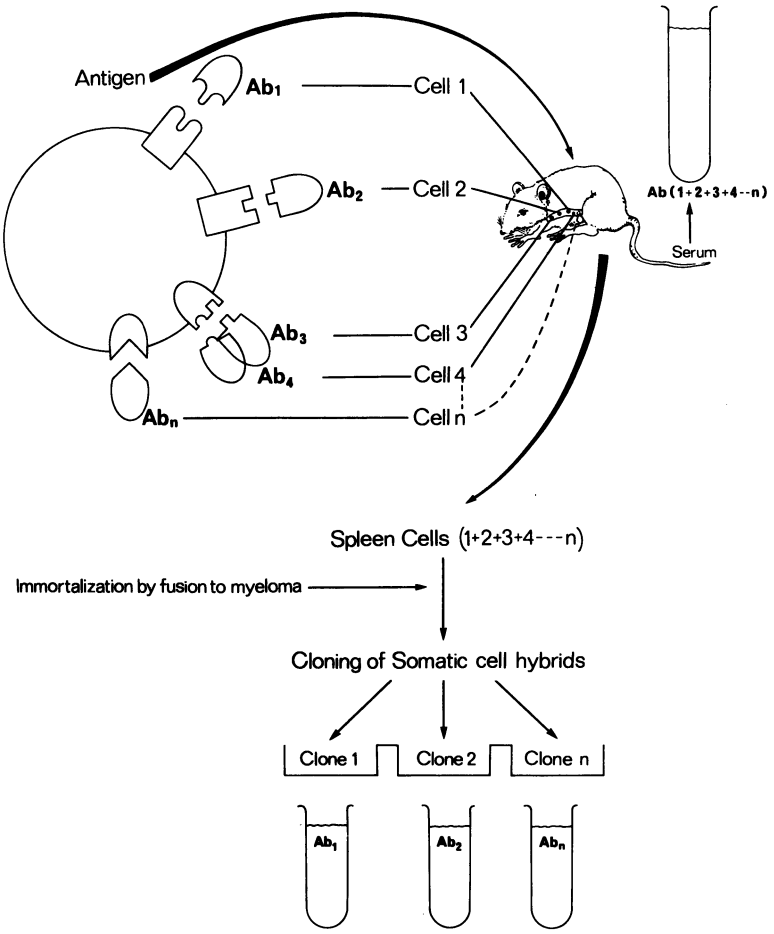

Fig. 3. Antibody production by animals and by hybrid myelomas. Isolated clones from hybrid myeloma fusions permit the preparation of pure monospecific antibodies directed to single antigenic determinants, starting from a complex mixture of antigens.

By fusion with a myeloma it is possible to fix the transient expression of individual cells in the form of permanent hybrid cultures. Such cultures can be subjected to the usual techniques of cell culturing and can therefore be purified and stabilized by subcloning (Fig. 3). By segregation analysis, subclones can be derived so that the myeloma components which do not contribute to the antibody activity can be eliminated (Köhler & Milstein 1976; Milstein *et al.* 1977). The end-product is a collection of permanent lines, each of them producing a different monoclonal antibody.

DIFFERENTIATION ANTIGENS FROM RATS, HUMANS AND MICE

The above type of approach to the detection of differentiation antigens was first used to derive mouse antibodies to rat cell membranes (Williams *et al.* 1977) and to human tonsil cells (Barnstable *et al.* 1978). A number of clones have been established and their characteristics are listed in Table 2; full details can be found in the references just cited.

In recent experiments monoclonal antibodies to mouse differentiation antigens have been produced. About 20 differentiation antigens in the mouse have been described by conventional procedures.

TABLE 2

Monoclonal antibodies to cell surface antigens in rat and man

Clone	*Antigenic target*	*Tissue*	*Cellular recognition*	*Antibody*
		Mouse anti-rat[a]		
W3/13		Lymphoid, bone marrow, brain	T cells; myeloid (?)	IgG1
W3/25 HL		Lymphoid	T cell subset (including[b] helper and GVH functions)	IgG1
W3/15 HLK		Blood, bone marrow, thymus	Red cells, erythroid (?) precursors	IgG
W3/4 HLK		Lymphoid	B cells	IgM
		Mouse anti-human[c]		
W6/1 HLK	Blood group A	Blood	Erythrocytes	IgM
W6/32	HLA	Widespread	Widespread	IgG2
W6/34 HLK	Controlled by chromosome 11 (carbohydrate ?)	Widespread	Widespread	IgG2

[a] Williams *et al.* (1977).
[b] White *et al.* (1978).
[c] Barnstable *et al.* (1978).
GVH: graft versus host.

It was interesting to obtain information on the frequency at which new antigens could be found by random collection of monoclonal antibodies prepared by xenogeneic immunization. Mouse spleen cells were fused to the mouse myeloma NSI/1-Ag4-1. Immediately after fusion, the cell population was divided into a large number of cultures. After a couple of weeks hybrid lines grew in all the cultures and the presence of antibody in the supernatants was tested by mixing the spent culture medium with target cells (mouse spleen cells in this case). The presence of bound rat antibodies was detected by a ^{125}I-labelled anti-rat Ig in almost all the cultures (Fig. 4). Since there were so many positive cultures we grew them further while we were freezing stocks for later studies. The most vigorous clones were in this way allowed to dominate each culture and 10 different hybrid clones were randomly purified and studied. Some of the cloned antibodies gave very low binding to spleen cells. It was

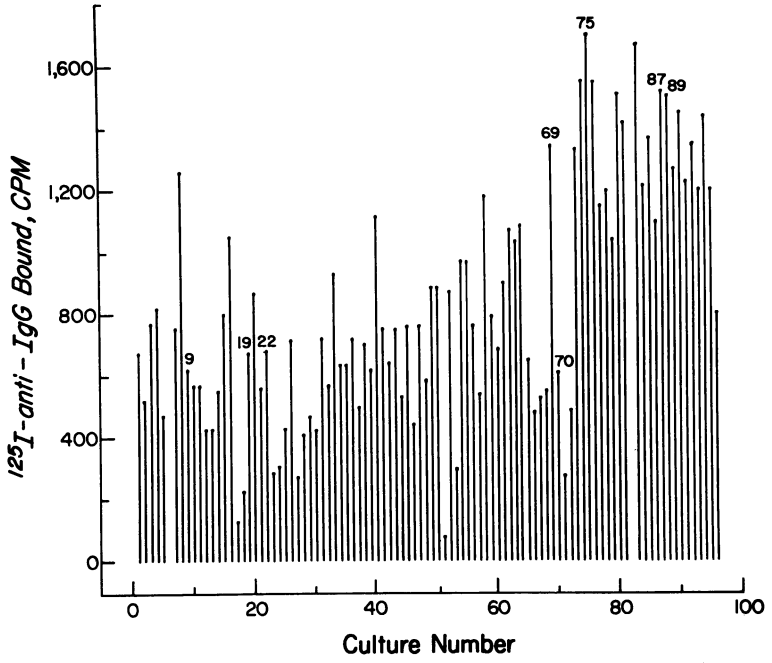

Fig. 4. Spleen cell binding activity of rat anti-mouse hybrid myeloma supernatants. Numbers refer to cultures from which hybrid clones were derived in this study.

only when we started to use panels of tumour cells that their true interest began to emerge (Stern *et al.* 1978; Springer *et al.* 1978*a*).

Table 3 summarizes the properties of the isolated antibodies and their antigenic targets. The table shows that the 10 monoclonal antibodies can be classified into four groups. Within each group, differences between the antibodies themselves can be demonstrated by several properties, especially by the electrophoretic properties of the isolated chains (Springer *et al.* 1978*a*). All of the antigens are differentiation antigens. None had been previously recognized by allogeneic immunization methods although one (reacting with M1/9.3 and M1/89.18) may be the same as a major iodinated membrane component observed by chemical and immunological methods (Trowbridge & Mazauskas 1976). The Forssman antigen is a heterophile antigen whose existence has been known for a long time (Humphrey & White 1963). The

TABLE 3

Rat monoclonal antibodies to mouse differentiation antigens

Clone	*Antibody*	*Cellular recognition*	*Antigen*	*Stability of antigen at 120°C*
M1/9.3 M1/89.18	IgG IgG	White cells	210 000 mol. wt.[a]	Unstable
M1/70	IgG	Macrophages and precursors	190 000 mol. wt.[a] 105 000 mol. wt.	Unstable
M1/75	IgG	Mouse RBC. Not on thymocytes	No iodinated component[a,b]	Stable
M1/69 M1/22.54 M1/89.1 M1/9.47	IgG IgG IgG IgG	Mouse RBC and most leucocytes. Thymocytes but not peripheral T cells.		
M1/22.25 M1/87	IgM IgM	Sheep RBC but not mouse RBC. Mouse teratocarcinomas. Minor cell subpopulations and early embryos[c]	Forssman	Stable

[a] The immuno precipitates were analysed by sodium dodecylsulphate—polyacrylamide gel electrophoresis.

[b] This group of antibodies compete between themselves for binding to mouse red blood cells (mouse RBC).

[c] Stern *et al.* (1978); Willison & Stern (1978).

other two have a very interesting cellular distribution (Springer *et al.* 1978*b* and unpublished). One of them—M1/70.15—appears to be present in macrophages and their precursors. Interesting relationships between the two can be deduced, in that they are expressed in a quantitatively inverse manner in the macrophage and its precursors. Thus differentiation from monocyte to peritoneal exudate macrophage is accompanied by a dramatic increase in the antigen recognized by M1/70, whereas the reverse appears true for M1/69.16. It appears, therefore, that the random derivation of monoclonal xenogeneic antibodies is a profitable approach to the detection of new antigenic surface components. A comparison of the ^{125}I-labelled membrane components precipitated by the antiserum from the rat which contributed the spleen for the fusion revealed many more bands than the composite of bands detected by the monoclonal antibodies (Fig. 5). Thus, the hybrid clones so far isolated in the experiment described represent only a few out of many clones responding to the immunization.

The use of monoclonal antibodies introduces a more reliable tool for studies of the quantitative expression of differentiation antigens. Instruments such as the fluorescent activated cell sorter (Loken & Herzenberg 1975) can measure the fluorescence intensity of large numbers of individual cells and permit a quantitative comparison of the antigenic expression in each cell. At the same time cell size can be measured by light scattering.

A simultaneous display of these two parameters introduces a new dimension to the analysis of cell subpopulations. This is well illustrated by Fig. 6 where distinct subpopulations are clearly defined by the monoclonal antibodies in a two-dimensional map or cellular fingerprint. Such cellular fingerprints may be of general use in the characterization of antigenic targets and antigenic variation as well as in defining new cell subpopulations.

W3/13 was found on thoracic duct T cells but not B cells, in a subpopulation (probably myeloid) of bone marrow cells and in brain (Table 2). M1/22.25 is expressed in a small subpopulation of spleen cells (apparently normoblasts), very strongly on teratocarcinomas and in four-day mouse embryos but not at earlier stages (Table 3). M1/69 is expressed in most leucocytes and thymocytes but not in closely related peripheral T cells. These antigens are therefore detected by monoclonal antibodies in different subpopulations and often unrelated populations of cells. The cell surface antigens of an animal may therefore be commonly expressed in different cell populations as a mosaic, the pattern of the mosaic being more characteristic of the individual cell type than the individual antigens themselves. This type of expression emphasizes the importance of the hybrid myeloma approach and the limitations of the absorption of antisera with tissues or cells different from the immunizing one.

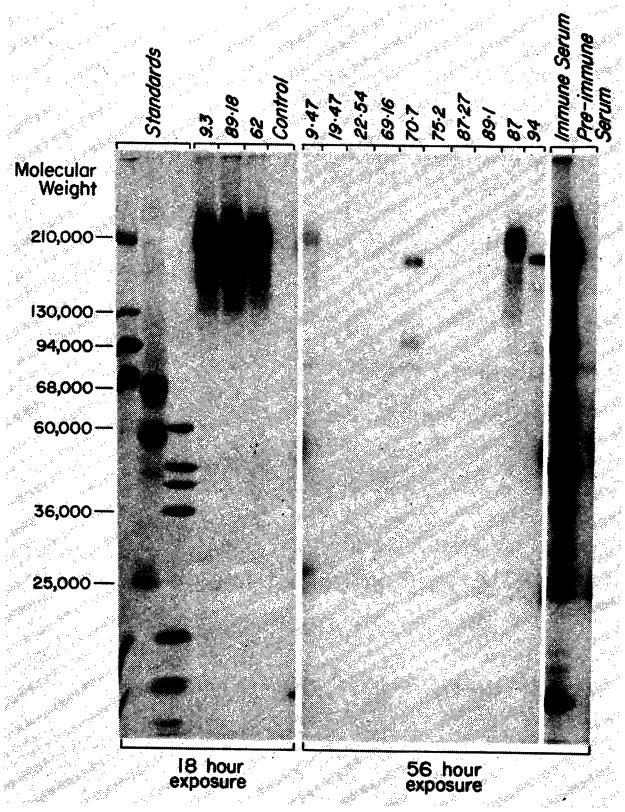

Fig. 5. Surface molecules precipitated by antibodies in the serum of the spleen (used for fusion) donor animal, in culture supernatants before cloning and in monoclonal supernatants. Concanavalin A-stimulated spleen cells were surface-labelled by lactoperoxidase and ^{125}I, solubilized with Triton X-100 and precleared by precipitation with anti-rat IgG. The subsequent specific immuno-precipitates were analysed by sodium dodecyl sulphate—polyacrylamide gel electrophoresis after reduction. Numbers refer to culture supernatants from Fig. 4, and individual clones are indicated by numbers after the dot.

FUSION PARTNERS AND THE EXPRESSION OF DIFFERENTIATED FUNCTIONS

One aspect of the hybridization experiments puzzled us from the very beginning. The desired hybrid myelomas were much easier to derive than we had thought. It soon became obvious that this was due to some form of selection or enrichment of the hybrids which expressed the antibody-secretion phenotype (Köhler & Milstein 1975, 1976). While the proportion of cells that secrete immunoglobulin is about 5% of the spleen cells used for hybridization, the proportion of hybrid clones derived that secrete immunoglobulin is well

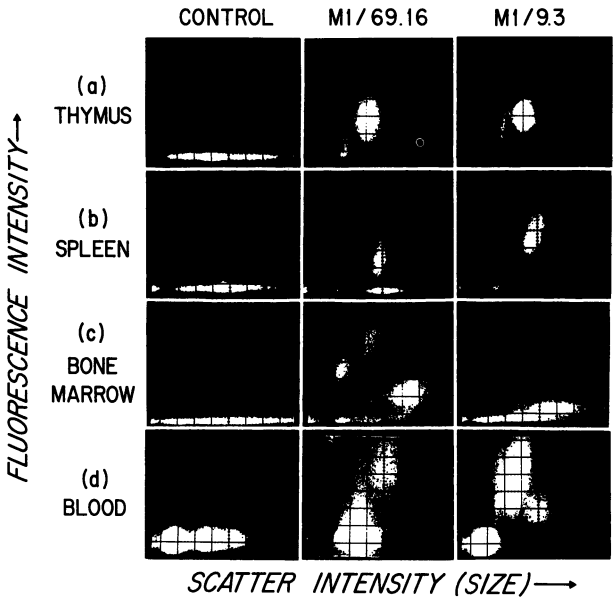

Fig. 6. Cellular fingerprints. Cell suspensions were depleted of red cells by Isopaque-Ficoll sedimentation and labelled with monoclonal culture supernatants followed by fluorescent anti-rat IgG.

over 50% (Table 4). Furthermore, if the mouse was immunized with SRBC, less than 1% of the spleen cells would secrete anti-SRBC, while the proportion of hybrid clones secreting anti-SRBC was 10%. In both cases the enrichment factor was about 10%.

The parental cell used for hybridization was a myeloma. However, when other parental cell types were used, the results were dramatically different. In other words, spleen cell hybridization to either a myeloma line or a T lymphoma line results in the selective expression of the spleen antibody in the first case and no expression of such a phenotype in the second. Conversely, the expression of the T cell antigen Thy-1 is selectively recovered from the spleen in the form of a permanent hybrid line when a T lymphoma, but not a myeloma, is used as the fusion partner (Table 4). This selective recovery of function may in part be due to an incompatibility of phenotypes since a fusion between a T lymphoma and a myeloma resulted in the suppression of the Thy-1 in the resulting hybrids. But that this is not the only explanation is suggested by the fact that the myeloma function (Ig secretion) was retained (Köhler *et al.* 1977*a*).

TABLE 4

Selective fixation of differentiated functions in established hybrids

Parental and hybrid lines	*Cell phenotype*			
	Ig secreted		*Thy-1 surface antigen*	
	Parental myeloma	*Other Ig*	*Thy-1, 1*	*Thy-1, 2*
X63 (myeloma)	>95%	0	0	0
(X63 × spleen) hybrids[a]	>90%	~65%	0	0
Spleen	0	~ 5%	0	~40%
(BW × spleen) hybrids[b]	0	0	>90%	~70%
BW (T lymphoma)	0	0	>95%	0

0 means none detected so far.
The spleens used were of an appropriate mouse strain. Further details in original references.
[a] Köhler *et al.* (1977*a*)
[b] Goldsby *et al.* (1977); Hämmerling (1977); Köhler *et al.* (1977*b*).

FUTURE PROSPECTS

The technique and the results described here seem to offer new vistas and some promising prospects in fundamental as well as applied aspects of human biology and human genetics (Table 5). In particular it appears worth while to explore the general use of cell fusion techniques for the recovery of transient differentiated functions in the form of permanent tissue culture lines. The results so far obtained strongly support our suggestion (Köhler *et al.* 1977*a*) that for the recovery of a transient differentiated function it is important to use cell lines phenotypically similar to the ones carrying the property in question.

TABLE 5

Some future prospects

1.	Anti-viral antibodies (for diagnosis and therapy)
2.	World standard reagents for organ transplantation
3.	Diagnostic reagents for clinical biochemistry
4.	Biochemistry and genetics of cell surfaces
5.	Affinity chromatography
6.	Fixation of other differentiated functions in permanent cell lines (T cells, hormone production, etc.)

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Discussion

Koprowski: We were able to produce intraspecies hybridomas by fusing P3 × 63Ag8 mouse myeloma cells (Köhler & Milstein 1975) with splenocytes from mice immunized against viral antigens or human tumour cells grown in culture (Koprowski *et al.* 1978*a*) (Table 1). In addition, we were successful in producing interspecies hybridomas between P3 × 63Ag8 cells and human lymphocytes obtained from either the blood or cerebrospinal fluid of patients suffering from a CNS disease (Koprowski *et al.* 1978*a*). The human × mouse hybridomas were found to segregate into clones that produce human IgM and clones that do not. It will thus be possible to assign the gene(s) for human immunoglobulin production to specific chromosomes.

Fusion of influenza (Gerhard *et al.* 1978), rabies (Wiktor & Koprowski 1978) or parainfluenza virus (Gerhard *et al.* 1978)-primed mouse spleen cells with P3 × 63Ag8 cells frequently and reproducibly resulted in the production of somatic cell hybrids. When 1.6×10^6 splenocytes of mice immunized with the PR8 strain of influenza A virus were fused with mouse myeloma cells, 100% of the hybrids secreted anti-influenza antibody. Through a gradual decrease in the number of influenza-primed splenocytes in the fusion experiments, it was possible to determine that antigenically stimulated B cells have a distinctive advantage over non-stimulated B cells in forming hybrids with mouse myeloma cells.

Antibody-producing hybridomas could be maintained indefinitely either in tissue culture or in ascitic form in BALB/c mice (Koprowski *et al.* 1977), and in the course of *in vitro* or *in vivo* passage neither the secretion nor the reactivity of the antibody changed significantly (Gerhard *et al.* 1978). Since in several hybridomas the IgG 3 fraction exhibited anti-influenza activity, it was possible to purify the hybridoma antibody for production of anti-idiotype antiserum in order to compare the idiotypic determinants of antibodies produced by hybridomas with those produced *in vivo* (Gerhard *et al.* 1978). The antigenic drifts characteristic for influenza A viruses were investigated through the study of variants of the virus selected after passage of the parental virus in the presence of monoclonal hybridoma antibody (Gerhard & Webster 1978). The results suggested that a single amino acid substitution may account for the antigenic modifications on the haemagglutinin molecule. Finally, interaction between monoclonal anti-influenza antibodies and a panel of influenza viruses provided a tool for rapid determination of binding constants for antibody–antigen reactions (Frankel & Gerhard 1978).

Until now only minor antigenic differences have been described for rabies virus strains. The interaction of monoclonal antibodies produced by hybridomas with various strains of rabies virus allowed us to detect marked differences in antigenic determinants within strains of fixed and street rabies virus (Wiktor & Koprowski 1978). Furthermore, since it was possible to produce monoclonal antibodies reacting only with nucleocapsids or only with envelope proteins of the virus, this technique greatly facilitates biochemical characterization of isolated viral components. Hybridoma antibodies also protected mice against the lethal effect of the virus.

Until now, transforming proteins of cells transformed by oncogenic viruses such as SV40 or polyoma could not be identified or biochemically characterized because of their cross-reactivities. Monoclonal antibodies produced by a hybridoma against an SV40 tumour (T) antigen showed specificities for the SV40 T antigen only and opened the way for its isolation in a pure form and its biochemical analysis (Martinis & Croce 1978).

Fusion of P3 × 63Ag8 cells with splenocytes from mice immunized against human melanoma produced hybridomas secreting antibodies that reacted only with melanoma tumours grown in culture or obtained from patients (Koprowski *et al.* 1978*a, b*). The hybridoma antibodies did not cross-react with normal cells (skin fibroblasts, liver cells, erythrocytes) obtained from the same patients who donated the melanoma cells (Steplewski *et al.* 1979). Antibody secreted by hybridoma cells maintained in diffusion chambers in the peritoneal cavity of nude mice suppressed growth of human melanoma implanted in these mice (Koprowski *et al.* 1978*b*).

TABLE 1 (Koprowski)

Profiles of monoclonal antibodies produced by hybridomas

Hybridomas obtained by fusion of P3 × 63Ag8 mouse myeloma with:													
Splenocytes of mice immunized against:													
Viral antigens								*Tumour antigens*				*Human lymphocytes obtained from:*	
Influenza A	Influenza B	Parainfluenza 1	Rabies	Herpes		Measles	SV40	SV40 trans-formed cells	Polyoma trans-formed cells	Human mela-noma	Human colo-rectal Ca	Blood[a]	CSF[b]
				Herpes simplex	Herpes canis								

CSF = cerebrospinal fluid from patients with CNS disease

[a] Producing human IgM

[b] Producing small amounts of human IgG

Fusion of P3 × 63Ag8 cells with splenocytes of mice immunized *twice* with human colorectal carcinoma cells resulted in the production of hybridomas (Koprowski *et al.* 1978*a*) that secreted antibodies cross-reacting with a number of antigenic determinants present on human cells derived from neoplastic or from normal tissues. However, when splenocytes were obtained from mice after only one immunization with colorectal carcinoma cells, it was possible to produce hybridoma antibodies that reacted only with colorectal carcinoma cells.

This brief survey of antibody profiles produced by hybridomas grown in tissue culture and studied at the Wistar Institute should end on a note of congratulation to Cesar Milstein for opening new vistas in immunology, cell biology and genetics.

Nossal: Anybody who starts using hybridoma antibodies may be a little disappointed with their strength in an immunofluorescent assay, for example. This requires a bit of thought. The molar concentration of antibodies can be enormously high in tissue culture hybridoma supernatants or in the sera of mice bearing the hybridoma tumours *in vivo*. At 1 in 10 000 or 1 in 100 000 dilution they can still react. However, at saturation they may appear to be rather weak reagents, giving less intense immunofluorescence than polyvalent antisera. If one is, for example, making an anti-human thymocyte serum in a rabbit there are two points to note if that is done in the conventional way, just through an immunization. First of all, the rabbit will recognize the foreignness of a number of different proteins and carbohydrates on the human cell surface. For that conceptually quite trivial reason, the antiserum that is made, which is a very heterogeneous population of antibody molecules, will have more molecules bound to the human cell surface at saturation.

But there is a more subtle point. Supposing you are talking about all of the antibodies made to a single molecule. Any protein is a mosaic of a very large number of antigenic determinants. In a conventional immunization, the sole factor limiting how many antibody molecules can be squeezed onto that single protein molecule will be steric hindrance. The protein could have 20 different antigenic determinants. But with monoclonal antibody, which is a pure protein directed against just one antigenic determinant, at saturation just one antibody molecule binds to each protein molecule on the cell surface.

There is a two-fold lesson to this. First of all, don't be disappointed if the fluorescent glow on your cell is rather weaker than you expected. Secondly, be sure to do your experiments at saturation and you will have a very beautiful quantitative technology for determining how many of the molecules being recognized are present on your cell.

Dr Milstein, your human T cell antigen looks extremely like TL in the

mouse, which is an MHC-linked antigen that appears on thymus lymphocytes but not peripheral T cells, and also is expressed in the thymic-type leukaemias. One would predict then that your marker would be present on human T cell leukaemic lymphocytes.

Milstein: In collaboration with A. McMichael and his colleagues in Oxford we have studied monoclonal antibodies produced by hybrids made with spleens of mice immunized with human thymocytes (McMichael *et al.* 1979). One of the hybrids has been cloned and the pure line is now well established, producing an antibody which is highly specific for human thymocytes. The antigen detected by this antibody, which we have named HTA.1 (human thymocyte antigen) is expressed in T cell leukaemias but not in all of them, and is not expressed in B cell leukaemias. (These tests were performed in collaboration with Dr G. Janossy from the Royal Free Hospital, London.) The expression of this antigen in normal thymocytes appears to be reciprocal to HLA in that thymocytes positive for one are negative for the other and vice versa. The antigen contains a component of about 45 000 mol.wt. In these properties it resembles the TL antigen of mice. However, no β_2-microglobulin component associated with it was detected, which seems to depart from the mice analogy, but there are some doubts about these results. In particular Dr A. Ziegler in my laboratory has strong reservations and wants to reinvestigate the problem.

I fully agree with Dr Nossal's comment on the use of monoclonal antibodies to quantitate the number of molecules of antigen per cell. This is something we have been doing with our reagents. In the case I have just been talking about, HTA.1 seems to have about 150 000 molecules per cell and is therefore a major surface component.

Bodmer: The published evidence for the β_2 association is in fact fairly weak. It may turn out that you are right and the others are wrong.

In the work we did together, two of the antibodies against the chromosome 11 determinants, W6.34 and W6.46, were very similar. Subsequently we found that sort of thing in other fusions. Sometimes perhaps one could get the same antibody in fusions because different cells from the same antibody clone in the spleen are being fused, and presumably in that case one would expect them, for example, to have the same isoelectric points.

I think there is a major distinction between looking at the carbohydrates, whether they are on the lipid or the protein, and looking at protein determinants. As you and others including ourselves find, a lot of these antibodies may be against sugar determinants. A lot of the things that give the cellular fingerprints may be those carbohydrates. One may expect to find that relatively minor perturbations of the biosynthesis of the sugar side chains may

produce these differences, and may have questionable biological significance. We don't know anything about the role of these sugars anyway, and of course there are well defined examples like the loss of ABO specificity on fibroblasts in tissue culture. Perhaps that sort of thing should be clearly distinguished from the expression of a given protein, which tends to be more definite: either it is in a particular class of cells or it isn't.

Harry Harris: Are you saying that if monoclonal antibodies against a glycoprotein are made, most of the antibodies would be against carbohydrates?

Bodmer: No. When one immunizes with whole cells or with membranes in xenogeneic immunizations, a substantial proportion of the antibodies obtained are apparently against sugar determinants, probably on glycolipids. An important class of these determinants, as Don Marcus and others have shown, have quite distinctive tissue distributions.

When we immunize with purified glycoproteins, which we have done for example with HLA, we don't find antibodies to the sugars on them. We have a whole collection of antibodies to HLA determinants, including a number which are polymorphic, by immunizing with purified glycoprotein. It is probably the sugars on the lipids that are the most antigenic. That is also the classical blood group experience.

Milstein: The response to carbohydrates in hetero immunization, from our experience so far, seems to be quite substantial. This is true not only when we immunize with membranes but also when we immunize with whole cells. In the case I described in my talk whole mouse cells were used as immunogen. Of 10 hybrid clones, seven seem to recognize what appears to be carbohydrate or lipopolysaccharide. We can't find protein bands in the gels, and they are thermostable, which is a classical property of carbohydrate antigens. Perhaps anti-carbohydrate differentiation antigens have been largely absent from previous studies using heterologous antisera because they require absorption with different cell types which may also include the antigen in minor subpopulations. If one was doing intraspecies immunizations one wouldn't find them because they are not polymorphic. The cloning of hybrid myelomas eliminates the problem.

Bodmer: We have found them immunizing with hybrids.

Klein: Is the class of antibody made by the hybridoma frozen or can it be switched?

Milstein: The class of antibody seems to be quite frozen. We could not switch from one to the other, even when we tried it in different ways (Milstein *et al.* 1977). One attempt was with a tumour which expressed both the specific anti-SRBC μ chain and the γ_1 chain from the parental myeloma, to see

whether we could switch from μ to γ antibodies. This was tested by suppressing the direct plaques (μ) with antimacroglobulin and developing indirect plaques to detect γ anti-SRBC activity. We tested 10^6 cells in this way (with appropriate controls) and they were negative. *In vitro* cellular switch must therefore occur at a frequency of, say, less than 1 in 10^6. If there is an error in switching in individual cells of the order of a few per cent we wouldn't know, but the cell itself doesn't switch in that way. We also injected the cells back into the animal but we couldn't switch them in that way either. Nor did ultraviolet irradiation work.

Gardner: Could this system be used to look at whether gene inactivation or repression is a particularly stable phenomenon in relation to determination in general? I don't find that the rarity of transdetermination detected in imaginal disks is very compelling. One could say that it is a problem not of the frequency of the event but of the frequency of detectability. Your system, with a very well-defined specificity in terms of the molecule that is being produced, and the possibility for enormous clonal expansion, might be ideal for looking at that particular problem.

Milstein: We don't know whether a translocation event or some DNA rearrangement is needed for the switch but it is definitely needed for the V-C combination. Whatever the mechanism of the switch, it appears to be extremely stable.

Gardner: If a hybrid is producing an antibody of a particular specificity and that clone is built up, is the specificity retained?

Milstein: The quick answer is yes. There is of course a certain loss of activity. We studied that earlier by looking at the clonal diversification of the myeloma line MOPC 21 and a certain frequency of irreversible loss of antibody gene expression was described. It is not excluded that this is due to chromosomal losses. Also there is a certain frequency of spontaneous mutations and that is where the mutants that I was talking about earlier came from.

Ruddle: So the stability of the hybrids is affected by the trivial loss of chromosomes?

Milstein: Absolutely; especially at the beginning. That is why multiple cloning is important in stabilizing the hybrids.

Peto: In the list of possible future uses why didn't you include tumour therapy? An incredible amount of very non-specific immunotherapy research is being done.

Milstein: I included diagnostic reagents. If I had put tumour therapy on the list I would have to deal with the press tomorrow!

Gurdon: How effectively does this procedure work with solid tissues? Most

of the cells you described were single cells. If you cut frozen sections does the increased specificity make it easier to detect markers?

Milstein: Yes, we have examples of that. It works well with solid tissues.

Jacob: I would like to return to the homology between the chromosomes of man and mouse and discuss the possibility of having something in the human like the *T* locus in the mouse. In the mouse, the H-2 complex is located on chromosome 17. On the same chromosome there is also a segment on which a number of mutations are known. These complex mutations have been detected because they affect the tail and for this reason are called *T*. Some are recessive *(t)* and some are dominant *(T)* for tail length. Most of them are recessive lethals. They also affect sperm formation in males. Many of the $+/t$ heterozygote males transfer the abnormal allele with a high frequency to the progeny: some 90% of the progeny inherit the *t* mutation. They also affect recombination on a chromosome segment from the centromere to further than the H-2 locus (for details, see Bennett 1975).

In humans, a disease which might recall some of the lesions due to the *t* anomalies in the mouse is spina bifida, a disease of the vertebral column in which the vertebrae do not close over the spinal cord. Some are widely open and are called 'aperta'; others are barely open and are called 'occulta'. There are many genetic and environmental causes of spina bifida. The work I am going to report was done in Paris by Marc Fellous, Joëlle Boué and Jacques Hors, who studied a family from Charleville, in northern France. In this family, a high incidence of disease of the sacrum is found, going from a complete absence of sacrum to a spina bifida occulta which can be detected only by X-rays. The disease appeared in a man who was the father of four children. These four children were affected and among 29 of his progeny, 19 were affected to various degrees.

In the mouse, the *T* locus is linked to the major histocompatibility complex, H-2. So the possibility of a linkage between the disease and HLA was investigated in detail (see Fig. 1). The two haplotypes of the man who had the original disease are unknown. It is unlikely, however, that this man was homozygous for the HLA region, the frequency of homozygotes in France being less than 1%. It can be seen on the figure that haplotype *d* is very frequently transmitted with the disease. Among 23 informative patients, 19 have an HLA haplotype *d*. Another marker which is known to be located on the same segment of chromosome 6, phosphoglucomutase-3 *(PGm3),* was also analysed. Among 12 informative patients, the allele $PGm3^2$ was found in 11 cases, again suggesting a linkage. In contrast, no linkage was observed between the disease and other markers such as ABO blood groups or Rh.

To interpret these data, one could propose the hypothesis that in this family

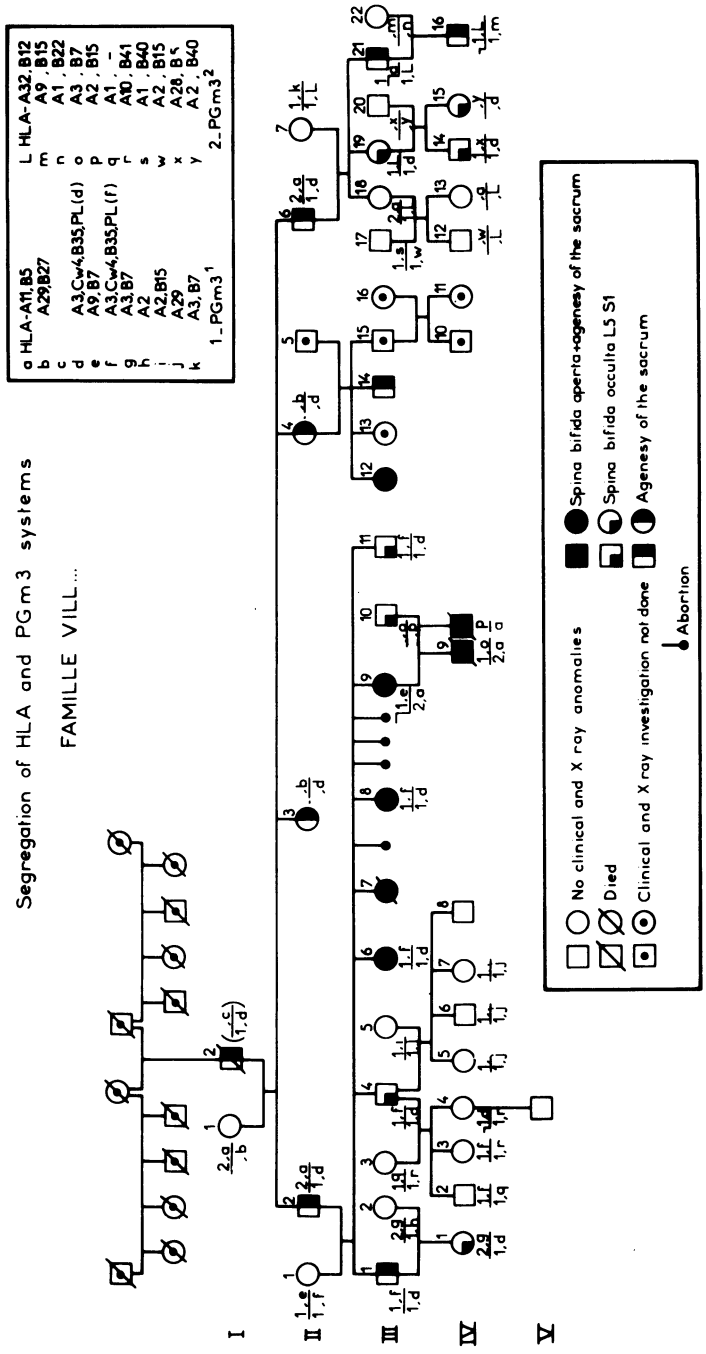

Fig. 1 (Jacob). Segregation of HLA and *PGm3* systems in a family from northern France.

the disease is due to a dominant gene, *Sp* (for spina bifida). This gene would be located on chromosome 6 and linked to HLA and *PGm3*. Although the figures are small, the order could be determined through the structure of recombinants, and especially children III_9 and III_{21}. III_9 is recombinant for the disease and HLA as well as for the disease and *PGm3*. III_{21} is recombinant between the disease and HLA but not *PGm3*.

The most likely order is therefore:

centromere—Sp—PGm3—HLA

Brenner: Is there any evidence for segregation distortion in man?

Jacob: Yes, there is an excess of disease in the family over random transmission. There is correlatively an excess of transmission of HLA haplotype *d*.

Brenner: Is there any HLA haplotype which shows segregation distortion?

Bodmer: Not as far as we know, but this would be an example of a rare mutant which is associated with segregation distortion. In general one would not expect HLA haplotypes to show that association. This family is extremely interesting.

Pontecorvo: Is it a damp area?

Jacob: Not especially. In this family the men were plasterers for three generations and then they changed their occupation.

Klein: What about the dominance relationships in the mouse *T* system?

Jacob: There are some mutations which are dominant and others which are recessive for the tail anomalies. They are recessive lethals.

Polani: This interesting family with sacral aplasia/hypoplasia reminded me of an observation by Degos and his colleagues (1974) on HLA in a tribe of Tuaregs in the Sahara Desert. Evidence from this tribe is pertinent to the question of the existence in humans of a situation analogous to that in the mouse, in respect to the *T* locus. In this highly inbred Tuareg isolate, the number of homozygotes for HLA haplotypes was significantly deficient. A possible explanation for this is that a locus on chromosome 6 of man, linked to HLA, may prevent homozygosity for HLA because homozygosity for one or more alleles at this linked locus could result in embryonic malformations such as those with some *T*-locus alleles in the mouse, and thus be embryo-lethal. Indirectly this would prevent homozygosis for the linked HLA alleles.

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