Proceedings of the National Academy of Sciences
Vol. 67, No. 3, pp. 1398–1403, November 1970

Selection of a Single Antibody-Forming Cell Clone and Its Propagation in Syngeneic Mice

B. A. Askonas, A. R. Williamson, and B. E. G. Wright

NATIONAL INSTITUTE FOR MEDICAL RESEARCH, LONDON, N.W. 7

Communicated by Sir Peter Medawar, August 6, 1970

Abstract. A single antibody-forming cell clone has been selected from primed mice by sequential transfer of limited numbers of spleen cells into irradiated syngeneic mice. The original spleen cell donors had been immunized with dinitrophenylated bovine gamma globulin. Specific antibody molecules in sera of recipient mice were separated by isoelectric focusing on polyacrylamide gels and visualized by ^{131}I-hapten binding and autoradiography. This method provided a marker for antibody-forming cells derived from a single cell clone. This report describes the history of one clone of cells (E9) producing IgG antibody to dinitrophenyl. Clone E9 is long-lived and has been maintained for five transplant generations (over 6 months) by serial transfer of spleen cells into irradiated syngeneic mice. Clone E9 has the following properties: (1) Antibody production strictly depends on antigen, presented either *in vivo* or *in vitro;* (2) Induction of E9 anti-dinitrophenyl shows specificity for the carrier protein; (3) Antibody is produced in amounts (2–3 mg/ml serum) comparable with myeloma-protein production by murine plasmacytomas; (4) In the absence of antigen, memory cells have a lifetime exceeding 28 days.

Transfer of limited numbers of primed lymphoid cells to irradiated syngeneic hosts has great potential for cell cloning in spleens.[1–3] Development of a simple direct assay for antibody heterogeneity has enabled us to use serial transfer to irradiated hosts as a selection system for single clones of antibody-forming cells; i.e., a family of cells with identical immunoglobulin products, derived from a single cell or identical cells.

Antibody heterogeneity was analyzed by high-resolution isoelectric focusing in thin layers of polyacrylamide gel, followed by specific detection of antibody molecules with radioactive hapten (manuscript in preparation). In using this assay to select for single-cell clones we were guided by the demonstration that the isoelectric spectrum of serum G-myeloma protein consists not of one, but a distinctive pattern of several protein components; such heterogeneity arises by postsynthetic modification of the homogeneous immunoglobulin formed by the myeloma cells.[4]

Methods. Isoelectric (pI) spectra of dinitrophenyl (Dnp) binding antibodies: Samples (20–40 μl) of serum were analyzed by isoelectric focusing with Ampholine carrier ampholytes (pH range 5–8) in thin layers of 5% polyacrylamide gel.[5] After focusing for 24 hr (final potential 500 V), the surface of the gel was coated with a 1 μM solution of [^{131}I]-α-*N*-(3,5-diiodo-4-hydroxyphenacetyl)-ϵ-*N*-(2,4-dinitrophenyl)-L-lysine (abbre-

viated [^{131}I]DDL). Development at 37°C for 15 min was followed by fixing in 18% (w/v) Na_2SO_4 solution. Amounts of Ig greater than 10 μg per band were visible at this stage. The gel was washed, dried, and a contact autoradiograph made on Ilfex x-ray film to reveal the pI spectrum of the antibodies. Protein components were subsequently visualized by staining with coomassie brilliant blue. Full details of this method will be published elsewhere. 19S IgM does not enter a 5% gel and is therefore excluded from analysis.

Cloning of antibody-forming cells: All experiments were carried out in female CBA mice (20–25 g) kept as an inbred strain at the National Institute for Medical Research. Mice serving as spleen cell donors were primed by a single intraperitoneal injection of $(Dnp)_9$-BGG (100 μg) and *Hemophilus pertussis* vaccine (2×10^8 organisms). After 3–5 months [at a time when these mice could give a strong secondary response to (Dnp)-BGG] a spleen cell suspension was prepared from a single donor and $1–5 \times 10^6$ cells/mouse were transferred intravenously into a series of irradiated (660 rads) syngeneic hosts together with 10–100 μg (Dnp)-BGG in saline. Recipient mice were bled 9–11 days later and the pI spectrum of serum anti-Dnp was analyzed. After a booster dose of (Dnp)-BGG, mice were selected to serve as spleen donors on the basis of restricted antibody pI spectra and high antibody production. For maintenance of selected clones $4–5 \times 10^6$ spleen cells were serially transferred intravenously into irradiated hosts at different time intervals (12–65 days) after antigen contact.

Results. The pI spectrum of IgG produced by a single plasma cell clone: It is well established that individual plasma cell tumour lines produce a single molecular species of Ig which undergoes alterations in charge properties after synthesis.[4] Methods affording high resolution, such as the focusing technique employed here, show that a G-myeloma protein as it exists in serum exhibits not one, but several protein bands. A typical pI spectrum (e.g. mouse G_{2a}-myeloma protein 5563, Fig. 1*a*) consists of a series of protein lines isoelectric at intervals of about

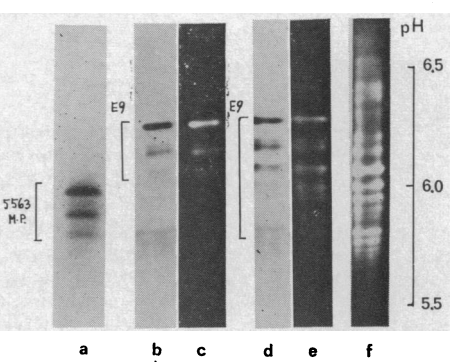

FIG. 1. Comparison of the pI spectra of antibodies to Dnp with G-myeloma protein. Isoelectric focusing was carried out using pH 5–8 Ampholine. (*a*) Purified mouse G_{2a}-myeloma protein 5563, protein bands stained with bromophenol blue. (*b*) Fresh serum from an E9 mouse (third transfer generation); protein bands visualized by precipitation with 18% (w/v) Na_2SO_4 after contact with [^{131}I]-DDL. (*c*) Autoradiograph of (*b*) showing the binding of [^{131}I]DDL by antibody molecules. (*d*) Serum from original mouse E9 (boosted) after it was stored frozen for 2 months and thawed several times for analysis. Protein bands visualized by Na_2SO_4 precipitation. (*e*) Autoradiograph of (*d*) showing uptake of [^{131}I]DDL by antibody molecules. (*f*) Secondary response: Serum anti-Dnp from CBA mouse, which received 10 μg (Dnp)BGG 3 months after priming with (Dnp)BGG.

0.1 pH units and decreasing in concentration with decreasing pI. E9 mouse antibody gives a similar type of spectrum (Fig. 1*b*,*c*). Such a pI spectrum is characteristic of the phenotype of a given cell clone and its progeny. The protein bands visualized by precipitation with Na_2SO_4 appear less complex than the antibody pI spectrum revealed by [^{131}I]DDL development and autoradiography. There is in fact an exact coincidence of major bands, but the extreme sensitivity of detection of the antibody accentuates the acidic minor components of the E9

spectrum. As is the case with myeloma proteins, storage of serum, and freezing and thawing, increases the number of protein bands (Fig. 1*d,e* compared with Fig. 1*b,c*). The functional homogeneity of E9 antibody is shown by hapten-binding measurement which give a heterogeneity index of 1.05 (ref. 6) with a K_a of the order of 10^{-7} liter mol^{-1} (ref. 7).

The simple pI spectra of E9 antibody may be contrasted with the heterogeneous spectrum of serum antibody to Dnp in a mouse 10 days after a second injection of (Dnp)-BGG (Fig. 1*f*). The complexity of antibody molecules defies analysis of the component overlapping pI spectra.

Clonal distribution of antibody-forming precursor cells in irradiated hosts: The complex antibody spectrum of the secondary response can be made more amenable to interpretation by distributing the precursor cells present in a primed spleen amongst a series of irradiated syngeneic recipients. An experiment of this type is illustrated in Fig. 2. Each of eight mice received intravenously 10^6 spleen cells (from a single donor primed 90 days earlier) and (Dnp)BGG (10 μg). More extensive series are under investigation. Analysis of the pI spectra in terms of line patterns characteristic of single-cell clones shows that the number of clones per recipient ranges from 0–3. The antibody pI spectra of individual host sera (Fig. 2) clearly show the restricted heterogeneity of antibody produced in such mice relative to a total secondary response (Fig. 1*f*).

History of E9 clone and some of its properties: A particular clone of cells (E9), that produces myeloma-like quantities of IgG antibody to Dnp, has been selected and maintained for over 6 months by serial transfer of spleen cells into irradiated syngeneic mice. E9 was derived from a CBA mouse primed with (Dnp)-BGG. 5×10^6 spleen cells (103 days after priming) were transferred to

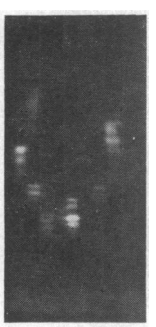

FIG. 2. Distribution of antibody-forming clones in irradiated mice reconstituted with 10^6 spleen cells from a donor mouse primed 3 months previously with (Dnp)-BGG. Autoradiograph shows pI spectra of anti-Dnp in sera of eight recipient mice 10 days after intravenous transfer of spleen cells with (Dnp)-BGG (10 μg).

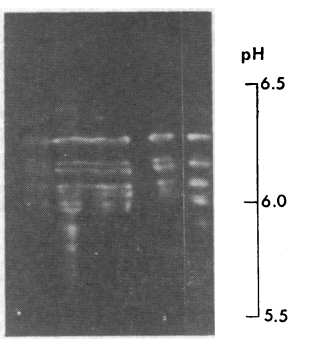

FIG. 3

FIG. 3. Autoradiograph, showing pI spectra of anti-Dnp present in selected sera from five transfer generations of E9 clone (covering a period of 145 days). Each serum is cross-referenced on the history chart (Fig. 4); the number of days indicate the time elapsed between original transfer of primed spleen cells to mouse E9 on day 0 and collection of serum from different host mice. Sera were analysed 10 days following injection of (Dnp)-BGG except for sample (*e*). (*a*) Mouse E9 (10 days) after initial transfer of primed spleen cells with (Dnp)-BGG (100 μg). (*b*) Mouse E9 (36 days) challenged with (Dnp)-BGG (10 μg). (*c*) Second transfer generation of clone E9 (46 days). (*d*) Third transfer generation (85 days); host received second generation cells but antigen was only given 18 days later (i.e., at 75 days). (*e*) Third generation (67 days); same host as (*d*) showing absence of antibody prior to antigen injection (*f*) Fourth transfer generation (102 days). (*g*) Fifth transfer generation (142 days).

each of 30 irradiated CBA mice, together with (Dnp)-BGG (100 μg). After 10 days, the serum of one recipient (numbered E9) contained anti-Dnp antibody with the simple pI spectrum shown in Fig. 3*a*. Such a pattern is representative of monoclonal antibody. A challenge with (Dnp)-BGG (10 μg) in saline 26 days after transfer resulted, 10 days later, in a greatly enhanced serum level of anti-Dnp (2.4 mg/ml) with the same pI spectrum as before (Fig. 3*b*, Fig. 1*d,e*) and only trace amounts of other antibody lines. This anti-Dnp spectrum, with a major band at pI 6.3, was attributed to a single clone.

Spleen cells (5×10^6) were transferred from mouse E9, with or without (Dnp)-BGG to 10 irradiated mice; only the six hosts receiving also (Dnp)-BGG produced E9 antibody; the amounts were comparable to those in the donor. In the absence of (Dnp)-BGG no detectable E9 antibody was formed; however, challenge with 10 μg of (Dnp)-BGG 2 weeks after spleen cell transfer (Fig. 4) or up to 28 days after cell transfer (not illustrated) resulted in levels of E9 antibody similar to those obtained when antigen and spleen cells were transferred simultaneously. Thus, in the absence of antigen, precursor memory cells of E9 have remained viable and are able to respond to antigen normally after 14–28 days.

A partial history of the serial transfer of clone E9 through five generations (over 5 months) is shown in Fig. 4 and anti-Dnp spectra of sera from each transfer

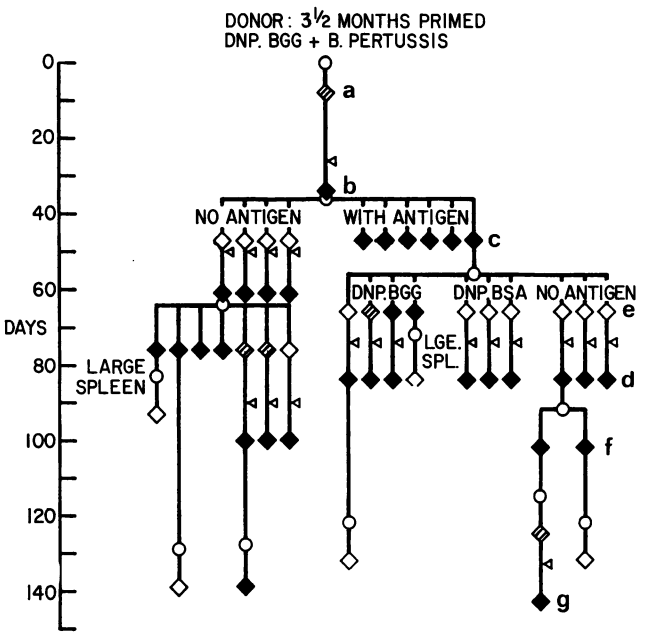

FIG. 4. A partial history of the serial transfer of E9 clone. The time scale refers to the time elapsed since the initial transfer of primed spleen cells to mouse E9 on day zero. All spleen cell transfers were made intravenously with (Dnp)-BGG except where otherwise indicated. O, time of transfer of spleen cells; ◇, recipient mouse producing no detectable antibody; ◆, mice producing E9 antibody at levels below 250 μg/ml; ◆, mice producing E9 antibody in excess of 250 μg/ml; ◁, intravenous injection of (Dnp)-BGG (10 μg) in saline. Letters *a–g* indicate the sera corresponding to the isoelectric spectra in Fig. 3. After the third generation, one symbol is representative of a series of recipients.

generation are illustrated in Fig. 3. Production of E9 antibody has remained entirely antigen dependent for 6 months, and shows some carrier specificity. At the third transfer generation, for example, spleen cells from a mouse producing large amounts of E9 antibody were transferred either in the absence of (Dnp)-BGG, or with Dnp on a heterologous carrier (Dnp-bovine serum albumin) which can precipitate E9 antibody. Only mice receiving (Dnp)-BGG produced E9 antibody (Fig. 4); however, challenge with (Dnp)-BGG after 18 days led to E9 antibody in all groups of animals. Induction of E9 antibody shows carrier specificity similar to that observed in heterogenous responses. Short contact (30 min) of E9 spleen cells with (Dnp)-BGG at room temperature *in vitro* sufficed for induction of E9 antibody, as tested in the fifth transfer generation.

After the third transfer generation, clone E9 could not be transferred consistently from donors producing E9 antibody. Some failures to transfer E9 could be attributed to the fact that 14–20 days after the syngeneic spleen cell transfer, splenomegaly occurred, unrelated to the presence or absence of antigen. Histological examination of the enlarged spleens (by Dr. B. Balfour) showed that neither bone marrow- nor thymus-dependent areas appeared to have been repopulated. However, after about 20 days splenomegaly was not observed and reconstitution of spleen improved. In later transfers, an interval of at least 4 weeks has been allowed between sequential cell transfers.

Serial transfer of spleen cells, with an interval of 65 days between generations and antigen contact, led to an apparent loss of the E9 clone, although chosen donors were still producing E9 antibody. By 65 days the spleen is thus depleted of E9 precursor memory cells either by death, exhaustive differentiation, or recirculation. A challenge with (Dnp)-BGG during this period, i.e. 38 days before cell transfer, maintained E9 clone precursors in the spleen.

Discussion. Our initial proposition, that the phenotypic marker of an antibody-forming cell clone would consist of a characteristic group of isoelectric lines, has been justified by the pI spectrum of IgG E9 antibody; this clearly resembles a G-myeloma protein spectrum and the E9 pattern has persisted through five generations of serial transfer. The Ig product of the cells has remained constant as observed for myeloma cells in mouse or man.[8] The complexity of the spectrum presumably arises from post-synthetic charge alterations of the homogeneous antibody produced by clone E9, by analogy with the full analysis of a mouse G_{2a}-myeloma protein.[4] Methodology with insufficient resolving power to reveal this post-synthetic heterogeneity is inadequate for studying the original heterogeneity of antibodies.

Combined with the isoelectric focusing assay for serum antibody heterogeneity, syngeneic spleen-cell transfer is a potent tool for selection of single clones of antibody-forming cells; selected clones can, by adjusting conditions, be maintained by serial transfer. During five transfers of clone E9, antibody production has remained strictly antigen dependent, though, as has been shown in other transfer of memory lymphocytes, brief contact with antigen *in vitro* will suffice.[9]

The idea that it is memory, in the form of precursor cells for clone E9, rather than autonomously dividing plasma cells producing E9 antibody, that are transferred between generations is supported by the fact that in the absence of antigen there is no detectable production of E9 antibody.

The transfer and half-life of memory lymphocytes have been studied previously;[10,11] "memory" then referred to the ability of lymphoid cells to give a secondary heterogeneous antibody response, which reflects turnover, recruitment, and development of different cell clones. Characterisation of the phenotype of the E9 antibody-forming clone has enabled us to study the longevity of memory cells and their progeny derived from a single cell clone. Our data shows the following:

(1) Administration of antigen up to 28 days after cell transfer in the absence of antigen (longest interval tested) elicited synthesis and secretion of E9 antibody. (2) E9 antibody production persists for at least 65 days in a single mouse. (3) Since its original detection, clone E9 has been propagated for over 6 months and promises to continue. At present our data do not distinguish between the life time of individual memory cells and overall duration of memory due to the antigen-dependent proliferation of precursor cells: similar levels of E9 antibody have been produced during the first few successive generations, although in each generation memory cells must be consumed by differentiation into plasma cells; low production of E9 antibody after spleen cell transfer with antigen can be increased with a second antigen dose.

The ability to differentiate into antibody secreting cells has been attributed to bone-marrow derived lymphocytes (reviewed in ref. 12); hence, we expect to be dealing with long-lived bone-marrow-derived cell clones. Antibodies with a given idiotype have persisted in rabbits for many months[13,14] but it is not yet clear whether a given idiotype is restricted to a single cell clone. In neonatal rabbits, antibodies with simple pI spectra are synthesized and can persist for at least 3 months.[6]

The method described in this report is potentially a general method for selecting single antibody-forming cell clones; it could be applied to any inbred line of animals and antigens of choice. It has proved possible to isolate other cell clones forming antibody to Dnp. This selection system provides a more systematic approach to the search for monoclonal antibodies than any previously used.[15-17]

Abbreviations: BGG, bovine gamma$_A$ globulin; Dnp, 2,4-dinitrophenyl; [^{131}I]DDL, ^{131}I-labeled α-*N*-(3,5-diiodo-4-hydroxyphenacetyl)-ϵ-*N*-(2,4-dinitrophenyl)-L-lysine.

[1] Kennedy, J. C., J. E. Till, L. Siminovitch, and E. A. McCullogh, *J. Immunol.*, **96**, 973 (1966).
[2] Playfair, J. H. L., B. W. Papermaster, and L. J. Cole, *Science*, **149**, 998 (1965).
[3] Klinman. N. R., *Immunochemistry*, **6**, 757 (1967).
[4] Awdeh, Z. L., A. R. Williamson, and B. A. Askonas, *Biochem. J.*, **116**, 241 (1970).
[5] Awdeh, Z. L., A. R. Williamson, and B. A. Askonas, *Nature*, **219**, 66 (1968).
[6] Montgomery, P. C., and A. R. Williamson, *Nature* (submitted for publication).
[7] Montgomery, P. C., unpublished; K_a determined by the method of Stupp, T. Yoshida and W. E. Paul, *J. Immunol.*, **103**, 625 (1969).
[8] Potter, M., and R. Liebermann, *Advan. Immunol.*, **7**, 92 (1967).
[9] Ellis, S. T., J. L. Gowans, and J. C. Howard, *Antibiot. Chemother.*, **15**, 40 (1969).
[10] Gowans, J. S., and J. W. Uhr, *J. Exp. Med.*, **124**, 1017 (1966).
[11] Celada, F., *J. Exp. Med.*, **125**, 199 (1967).
[12] Taylor, R. B., *Transplant. Rev.*, **1**, 114 (1969).
[13] Oudin, J., and M. Michel, *J. Exp. Med.*, **130**, 619 (1969).
[14] Dougharty, H., J. E. Hooper, A. B. MacDonald, and A. E. Nisonoff, *J. Exp. Med.*, **131**, 583 (1970).
[15] Haber, E., *Fed. Proc.*, **29**, 66 (1970).
[16] Eichmann, K., H. Lackland, L. Hood, and R. M. Krause, *J. Exp. Med.*, **131**, 207 (1970).
[17] Brenneman, L., and S. J. Singer, *Prod. Nat. Acad. Sci. USA*, **60**, 256 (1968).