groups that consisted of ten calls repeated at a call rate of 10 Hz. Synthetic calls were constructed by digitally adding sine waves. Calls were broadcast antiphonally with a Sony TCD-5M stereo cassette recorder, a stereo amplifier, and two ADS L-200-C speakers. The female was placed equidistant from each speaker (1.1 m); sound pressure level (SPL; relative to 2×10^5 dynes/cm^2) was 75 dB SPL at this point. The female was released by a remote device, stimulus broadcast began, and a phonotactic response was recorded if she approached to within 10 cm of one of the speakers. Each female was tested only once with each stimulus pair.

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Production of Stable Rabbit-Mouse Hybridomas That Secrete Rabbit mAb of Defined Specificity

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Inclusion of normal rabbit serum (NRS) in culture medium after interspecific fusion of hyperimmunized rabbit spleen cells with murine SP2/0 myeloma cells produced 271 rabbit-mouse hybridomas (RMHs) that secreted rabbit immunoglobulin against group A *Streptococcus* (GAS). Continued use of NRS-supplemented medium during cloning yielded stabilized monoclonal RMH lines that have secreted GAS-specific rabbit antibody at concentrations similar to murine hybridomas (3 to 8 micrograms per 10^6 cells per 24 hours), for over 4 months of culture in vitro. The use of NRS as a medium supplement during initial culture, cloning, and stabilization of RMHs enables production of considerably more specific rabbit monoclonal antibody (mAb)–secreting RMHs than have previously been reported.

HYBRIDOMA TECHNOLOGY HAS now reached a level where it is possible to produce mAbs against most antigens. Some immunogens, however, stimulate poor antibody responses in mice, and consequently high-affinity murine mAbs with certain epitope specificities can be difficult to obtain. These problems are reflected by the small numbers of GAS-specific mAb-secreting murine hybridomas that have been reported (*1–3*).

Rabbits, in contrast, produce high titers of high-affinity antibody when hyperimmunized with most immunogens, including streptococcal carbohydrate (*4*). The ability to produce rabbit mAbs could therefore overcome many of the disadvantages of murine systems. Myelomas, however, are unknown in rabbits, and transformation of rabbit B cells in vitro with viruses has proved difficult (*5*).

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RMHs have been produced (*6*) but, because of the instability of rabbit chromosomes in RMH cells, only cell lines that secrete a rabbit light (L) or heavy (H) chain, not an intact rabbit immunoglobulin, have been described. Kuo *et al.* (*7*), however, succeeded in producing RMH cell lines that secreted intact rabbit H and L chain immunoglobulins (Igs) but only obtained three stable clones from four different fusions.

One of us (T.J.G.R.) previously reported production of stable bovine-murine (*8*) and porcine-murine (*9*) hybridomas that secrete bovine or porcine mAb of defined specificities. We therefore applied these procedures in producing stable RMHs that secrete rabbit mAb specific to group A streptococcal carbohydrate.

A female New Zealand White rabbit was hyperimmunized with a nitrous acid extract (*10*) of group A *Streptococcus pyogenes* cells (American Type Culture Collection 19615) until its serum gave a high antibody titer in a GAS-specific enzyme-linked immunosorbent assay (ELISA) (*11*). We prepared RMHs by polyethylene glycol–mediated fusion (*12*) of spleen cells from this rabbit with murine SP2/0-Ag14 cells (*13*), using the method described by Gefter *et al.* (*14*). After fusion, cells were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) (*15*).

The RMH culture supernatants were screened for the presence of GAS-specific rabbit antibody by an ELISA (*11*). Selected positive culture wells were cloned by limiting dilution until stabilized, and resulting monoclonal hybridomas that secreted GAS-specific rabbit antibody were stored in liquid nitrogen. Rabbit mAbs selected for further study were produced by first adapting the appropriate RMH lines to grow in DMEM supplemented with fetal calf serum (FCS) [that is, in the absence of normal rabbit serum (NRS)]. Rabbit antibody was

Table 1. The effect of serum and feeder cell type on the outcome of fusion between hyperimmune rabbit spleen cells and murine SP2/0 plasmacytoma cells.

Feeder cells	Appearance of cultures on day 4 after fusion	Number of wells positive in initial ELISA*	Cell growth on day 17 after fusion
	Cell fusion A; 15% FCS		
Splenocytes and thymocytes (*18*)	Multiple RMH in every well, most at 16-cell stage	13% (40/300)	Massive fibroblastic cell growth, outgrowing hybridomas
Peritoneal exudate cells (*18*)	Multiple RMH in every well, most at 16-cell stage	30% (18/60)	Few areas of fibroblastic cell growth; good hybridoma growth
	Cell fusion B; 15% NRS		
Splenocytes and thymocytes	Many viable cells in every well, but no cell division	55% (159/288)	Minimal fibroblastic cell growth; good hybridoma growth easily observed
Peritoneal exudate cells	Many viable cells in every well, but no cell division	90% (54/60)	Minimal fibroblastic cell growth; good hybridoma growth easily observed

*Initial ELISA screening for GAS-reactive wells was performed on day 17 after fusion.

then purified from these RMH culture supernatants on a Sepharose column with an anti-rabbit IgG immunosorbent (*16*).

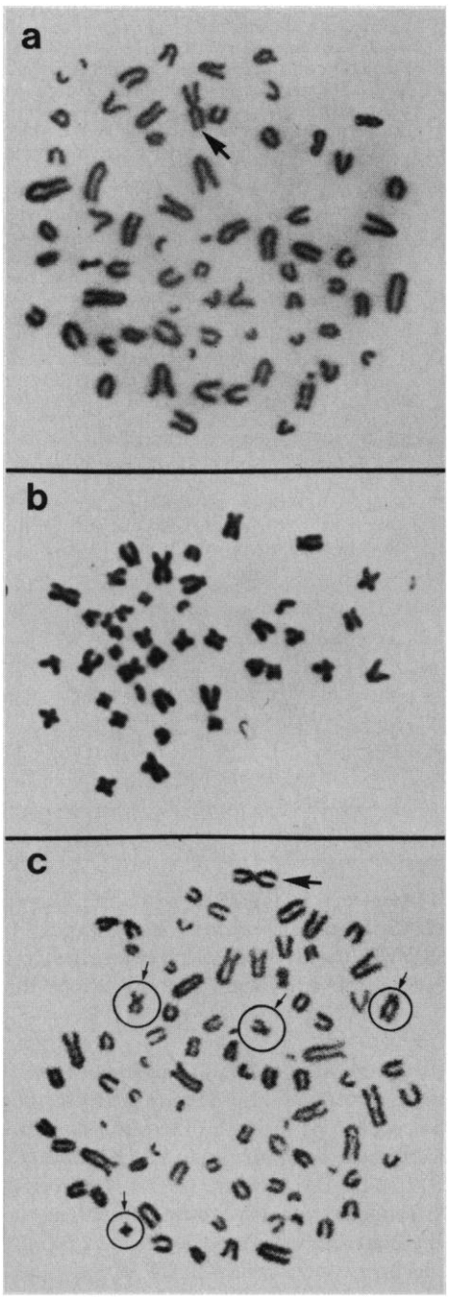

Fig. 1. Karyotyping of SP2/0-Ag14, rabbit, and RMH cells. Cells were exposed to hypotonic treatment in 0.075*M* KCl for 30 min, fixed in acetic acid–methanol solution, spread on glass microscope slides, and then stained with Giemsa stain. (**a**) SP2/0-Ag14 murine plasmacytoma cell showing acrocentric chromosomes and one large metacentric chromosome (large arrow) (average $2n = 65$). (**b**) Normal rabbit leucocyte showing metacentric chromosomes (average $2n = 44$). (**c**) RMH cell showing mouse acrocentric chromosomes, one large mouse metacentric chromosome (large arrow), and four rabbit metacentric chromosomes (circles and arrows). (Average $2n = 69$ at day 64 after cell fusion.)

The cells produced by fusion of rabbit spleen cells with murine SP2/0-Ag14 plasmacytoma cells were resuspended in DMEM supplemented with either 15% FCS (fusion A) or 15% NRS (fusion B), and distributed into 96-well tissue culture plates (Costar) with feeder cells (*17*) (Table 1). Examination of plates 4 days after fusion indicated that hybridomas were growing in cultures supplemented with FCS far more rapidly than in cultures supplemented with NRS. Seventeen days after the fusion, culture supernatants from wells with actively growing hybridoma clones were screened for the presence of rabbit antibody against GAS by ELISA. In spite of our initial observation, cultures supplemented with NRS produced more positive wells than those supplemented with FCS (Table 1). Furthermore, cultures containing a feeder layer of peritoneal exudate cells (PECs) yielded more positive wells than those containing splenocytes and thymocytes. These observations strongly suggest that the presence of NRS and PECs in RMH cultures increased the number of clones secreting GAS-reactive rabbit antibody.

In view of the high total number of ELISA positive wells (271), we selected a small number of primary hybridomas for cloning, on the basis of GAS-ELISA reactivity (reduced to 111) and growth rate (reduced to 44). During passage prior to cloning, considerably more primary hybridomas from fusion A (98%) were discarded because of loss of antibody production or poor growth rates than from fusion B (32%). Of 44 primary RMHs cloned by limiting dilution, 41 were cloned and stabilized successfully. None of the clones from fusion A survived cloning, but 95% of those from fusion B were cloned and continued to secrete rabbit antibody. Of these 41 RMH cell lines, 28 (68%) cloned and stabilized successfully after one cloning cycle, 11 (95%) after two, and the remaining 2 (100%) after three. ELISAs done with polyvinyl chloride (PVC) plates coated with nitrous acid extracts of groups A, B, C, D, F, and G *Streptococci*, on culture supernatants from the 41 RMH lines, confirmed that all 41 lines secreted GAS-specific rabbit mAbs. ELISAs for determining the Ig class of the rabbit mAbs [where peroxidase conjugates specific for rabbit IgG (γ chain) and IgM (μ chain) and recrystallized 5-aminosalicylic acid chromogen and substrate (*18*) were substituted for the Ureiase reagents] showed that 39 RMH lines secreted rabbit IgG, whereas the other two secreted rabbit IgM.

The data suggest that RMHs cultured in the presence of NRS were far more stable in terms of rabbit antibody secretion than those cultured in the presence of FCS. To test this hypothesis, ten primary hybridomas were cloned in duplicate, one replicate in DMEM supplemented with 7.5% FCS and 7.5% NRS, the other with 15% FCS. Although nine out of these ten primary hybridomas were cloned successfully in medium containing FCS alone and continued to form rapidly growing colonies (Table 2), stabilization of rabbit antibody production was considerably more efficient in those cloned in the presence of NRS.

The efficiency of rabbit mAb production in vitro was studied with eight RMH lines. These lines have continued to secrete GAS-specific rabbit mAb during 4 months of continuous in vitro culture and through at least two cycles of being frozen in liquid nitrogen followed by thawing and culturing. Each line was adapted, after stabilization, to grow in DMEM containing 10% FCS. Rabbit mAb was affinity-purified from culture supernatants by use of the Sepharose anti-rabbit IgG immunoadsorbent column. All eight affinity-purified rabbit mAbs showed GAS-specificity in ELISA. The yields of rabbit Ig [calculated from OD_{280} (optical density) values] secreted ranged from 3.26 to 8.76 μg per 10^6 cells per 24 hours, with a mean of 5.34 μg per 10^6 cells per 24 hours.

Table 2. Importance of NRS as medium supplement for establishment of specific-antibody secreting RMHs. Ten primary RMH lines were cloned in duplicate, one replicate of each in DMEM supplemented with 7.5% FCS and 7.5% NRS (experiment 1), the other with 15% FCS (experiment 2). Plates were examined for clonal growth after 8 to 10 days, and supernatants from wells containing growing clones were screened for GAS-specific rabbit antibody by ELISA.

RMH line	Experiment	Number ELISA-positive wells*
B 102	1	100% (26/26)
	2	24% (5/21)
B 104	1	100% (31/31)
	2	0% (0/21)
B 107	1	83% (19/23)
	2	0% (0/36)
B 120	1	96% (25/26)
	2	0% (0/23)
B 130	1	86% (12/14)
	2	0% (0/34)
B 139	1	89% (33/37)
	2	14% (4/28)
B 157	1	68% (17/25)
	2	36% (10/28)
B 158	1	100% (31/31)
	2	29% (5/17)
B 172	1	100% (26/26)
	2	33% (7/21)
B 174	1	100% (10/10)
	2	0% (0/0)

*Percent positive (ELISA-positive wells/total wells screened).

These values are similar to what might be expected for murine mAb from murine hybridomas when cultured in vitro. The purified rabbit mAbs have been used for successful development of ELISA systems for GAS detection.

Karyotyping of one RMH (B52) was performed to ascertain the number of chromosomes present at various times after fusion. By 64 days after fusion, the mean chromosome number of this line ($2n = 69$) had stabilized to approximately the same as the parent SP2/0 cells ($2n = 65$), with a coefficient of variation (5.6%) less than half that of the SP2/0 cells (13.3%). The karyotypes of four other RMH lines were also examined 3 months after fusion and found to contain a similar number of chromosomes. The nuclei of these RMH lines appear to contain three to five rabbit metacentric chromosomes, in addition to their complement of mouse acrocentric chromosomes (Fig. 1).

We have developed optimized procedures for producing large numbers of stable RMHs that secrete complete rabbit Ig molecules with excellent specificity to the GAS immunogen used. These procedures include the use of the SP2/0-Ag14 myeloma line as fusion partner, culture of RMHs after fusion in the presence of NRS and PEC feeder cells, cloning of RMHs in medium containing NRS, and once stabilized, gradual adaptation of monoclonal RMH lines to grow in medium containing FCS rather than NRS. The ability to use spleen cells from specificity hyperimmunized rabbits for generation of large numbers of stable RMH lines that secrete rabbit mAb against these immunogens should allow production of new mAb species that, to date, could not be produced.

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11. In the ELISA we used PVC microtiter plates (Dynatech Laboratories) coated with an optimized dilution (in 0.1*M* carbonate-bicarbonate buffer, *p*H 9.6) of nitrous acid extract (*10*) from *Streptococcus* cells of groups A, B, C, D, F, or G (ATCC 19615, E13813, 12388, 19433, 12392, or 12394; recommended by ATCC as being type strains antigenically representative of groups A, B, C, D, F, and G, respectively). Positive cultures were detected with an optimized dilution of urease-conjugated sheep antibody to rabbit IgG (H and L chain specific) and urease substrate. They were confirmed to be secreting rabbit Ig by differential reactivity in ELISA using this conjugate and optimally diluted urease conjugated rabbit antibody to mouse $F(ab')_2$ (Ureiase system, Allelix Diagnostics). The specificities of both these conjugates for rabbit and mouse Ig in the ELISA was established by using titrations of GAS-reactive rabbit and mouse antisera. Control wells were used in which DMEM supplemented with FCS or NRS was substituted for RMH culture supernatant. The absorbance matrix at 590 nm of ELISA plate wells was read on a Multiscan ELISA reader (Flow Laboratories, Inglewood, CA) calibrated on appropriate control wells.
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15. Supplemented DMEM contained glucose (4.5 g/liter), sodium bicarbonate (2.8 g/liter), Hepes buffer (20 m*M*), glutamine (2 m*M*), sodium pyruvate (1 m*M*), penicillin (50 IU/ml), streptomycin (50 μg/ml), and the appropriate percentage of FCS (Bocknek Ltd., Rexdale, Ontario, Canada) or heat-inactivated NRS (produced in house).
16. The anti-rabbit IgG immunoadsorbent was prepared from affinity-purified goat antibody to rabbit IgG (H + L) (Jackson Immunoresearch Laboratories) coupled to CNBr Sepharose 4B (Pharmacia Canada Inc., Dorval, Quebec, Canada) at 2 mg of protein per gram of gel, and poured into a column on top of Sephadex G-25 (Pharmacia Canada Inc.) (*19*). The specificity of this immunoadsorbent column for rabbit Ig was shown by passing culture supernatant and ascitic fluid containing murine mAb against GAS through it, and eluting with 2*M* sodium thiocyanate under conditions identical to those used for RMH culture supernatants. ELISAs on these control eluates showed that they contained neither mouse nor rabbit antibody against GAS, proving that the immunoadsorbent column bound only rabbit Ig.
17. Feeder cells were prepared from normal BALB/c mice by the methods of S. Fuller, M. Takahashi, and J. G. R. Hurrell [in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Wiley Interscience, New York, 1987), chap. 11]. Splenocytes and thymocytes (mixed in a 1:1 ratio) were seeded at 10^6 cells per well, whereas peritoneal exudate cells were seeded at 10^4 cells per well.
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A New Specimen of *Archaeopteryx*

PETER WELLNHOFER

A new specimen of the primordial bird *Archaeopteryx* is reported from the Upper Jurassic Solnhofen Limestone of Bavaria. This "Solnhofen specimen" is the largest of now six skeletal specimens and shows close similarities with the London specimen. It is therefore assigned to *Archaeopteryx lithographica* Meyer. Clear impressions of the feather shafts of the left wing are preserved.

A NEW, WELL-PRESERVED SPECIMEN of *Archaeopteryx* from the Upper Jurassic Solnhofen Limestone (Lower Tithonian) was discovered in a private collection in Solnhofen, West Germany, in November 1987. It is the sixth skeletal specimen of this famous fossil after the London, Berlin, Maxberg, Haarlem, and Eichstätt specimens (*1*) [unless one follows the suggestion of Howgate (*2*) to separate the smallest one, the Eichstätt specimen, as a distinct genus]. The new example is the largest of all, about 10% larger than the London specimen. The new specimen is housed and exhibited in the Bürgermeister-Müller-Museum in Solnhofen, and is therefore announced here as the "Solnhofen specimen" of *Archaeopteryx* (Fig. 1).

Exact locality data are not available. According to the collector, an amateur, the specimen was found many years ago and no data were kept. After preliminary preparation, carried out only recently, he took it for a specimen of the small theropod dinosaur *Compsognathus*, rather than of *Archaeopteryx*. Although the quarry site is no longer known, it must have been located in the Eichstätt area where the Berlin and Eichstätt specimens were found in 1877 and 1951, respectively (*3*).

The skeleton is quite complete and in almost natural articulation. It is preserved on a slab (52 by 39 cm) of Solnhofen limestone of the so called "Fäule" facies, a soft marly limestone with a content of 10 to 20% clay minerals (*4*). Preservation of the dark brown bone is generally good, and detailed preparations by both mechanical

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