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Cytostatic drug sensitivity test for human multiple myeloma, measuring monoclonal immunoglobulin produced by bone marrow cells in vitro*, **

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Summary. An in vitro cytostatic drug sensitivity test for human multiple myeloma has been developed, predicting differences in sensitivity of the individual tumor to various anticancer drugs. Bone marrow preparations containing the tumor cells were incubated with cytostatic drugs and cultured for 10 days. Using an enzyme-linked immunosorbent assay we measured tumor products – monoclonal immunoglobulin and β_2-microglobulin – in the culture supernatants. The reduction of these products in vitro due to the drugs administered was compared with the patients' further clinical course during treatment with different standard cytostatic drug regimens. We found a predictive value of more than 80% for this easily performed test.

Introduction

In the chemotherapy of multiple myeloma a few well-established cytostatic drugs, mainly alkylating agents, are used when indicated [3, 6, 9]. Although many other agents have been suggested, very few have proved to be effective in this tumor [6]. An in vitro test method predicting the sensitivity of multiple myeloma tumor cells in vivo would be a useful tool in screening for promising new drugs. In addition, such a method might improve established chemotherapy, as primary and secondary resistance of the individual tumor to the drugs used in standard therapy protocols could be recognized before the patient was treated with potentially toxic agents. Chemotherapy regimens with positively proven effectiveness in large studies could then be adapted to the individual situation of a given patient.

Tumor stem cell assays with a high predictive value have already been described for this purpose [5, 7, 8, 12]. In these assays, tumor stem cell colonies are counted after in vitro growth in an appropriate culture system, requiring special conditions for cloning efficiency and highly specialized personel for correct and reproducible evaluation.

This is also needed for the nonclonogenic methods described for use in multiple myeloma [2, 16].

In short-term cultures of bone marrow cells in multiple myeloma patients, we have found spontaneous production of monoclonal immunoglobulins (mIg) detectable by an enzyme-linked immunosorbent assay (ELISA). Since this mIg is produced by tumor cells proliferating in vitro, a sensitivity test measuring this tumor product should have a predictive value comparable with results of the stem cell culture methods, but may allow the testing of more patients with a simpler technique. Because β_2-microglobulin has been described as a good marker for cell proliferation in multiple myeloma [1, 4], we also measured this protein as a second marker in the bone marrow culture supernatants.

Materials and methods

Patients. The group of patients tested was made up of 24 with multiple myeloma (MM), 9 of whom had been pretreated with cytostatic drugs, 8 patients with monoclonal gammopathy of undetermined significance (MGUS), 6 with non-Hodgkins or Hodgkin's lymphomas, 1 patient with renal insufficiency, and 1 patient with systemic lupus erythematosus (SLE). Bone marrow cells were obtained by Jamshidi biopsy performed for diagnostic purposes. Pretreated patients were tested at least 3 weeks after the last cytostatic drug treatment. MM patients were afterwards treated according to standard therapy protocols irrespective of the test results. The response to chemotherapy was evaluated according to criteria established by Durie and Salmon [13]: a reduction in the serum myeloma protein level by more than 25% was defined as partial remission (PR) and a reduction by more than 75% as complete remission (CR), while an increase by more than 25% was considered as progress (P).

Cultures of bone marrow cells. Samples of 8–20 ml bone marrow aspirate were obtained. The syringe contained 200 units Heparin Novo (Novo Industrie GmbH, Mainz). The bone marrow aspirate was diluted with an equal volume of RPMI 1640 (Flow Laboratories, Edingburgh). Mononuclear cells were separated on a Ficoll gradient (Seromed, Munich) by centrifugation (20 min, 1000 g, 20 °C) and washed three times in RPMI 1640.

Cells (16^6) were cultivated in round-bottomed plastic tubes (Falcon 3033 tissue culture tube: Falcon Plastics, Los Angeles) in 1 ml culture medium. The culture medium was RPMI 1640 (containing 2.0 g/l bicarbonate) supplemented with 10% inactivated fetal calf serum (Seromed, Munich) 1% L-glutamine (Flow Laboratories, Edinburgh), 100 µg

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streptomycin, and 100 units penicillin/ml (TC Penicillin-Streptomycin; Difco Laboratories, Detroit). For each test, double or tirplicate cultures containing the cytostatic drugs (see below), control cultures containing 50 µg puromycin (Serva, Heidelberg) for complete protein synthesis inhibition [10], and control cultures without any additives were set up. After 10 days' incubation at 37 °C in a 5% CO_2 atmosphere the culture supernatant was harvested and measured in an ELISA as described below.

Cytostatic drugs. The following cytostatic drugs were diluted with culture medium to give the indicated final culture concentrations and added to the cell cultures: Melphalan 0.015–0.15 µg/ml (Alkeran, 100 mg amp., Deutsche Wellcome GmbH, Burgwedel), 4-hydroperoxycyclophosphamide 0.5 µg/ml (provided by ASTA Werke AG, Bielefeld), vincristine 0.006 µg/ml (Vincristine liquid, 1 mg amp., Eli Lilly GmbH, Giessen), carmustine 0.15 µg/ml (Nitrumon, 100 mg amp., Siphar GmbH, Cologne), doxorubicin 0.015 µg/ml (Adriblastin, 10 mg amp., Farmitalia GmbH, Freiburg), vindesine 0.01 µg/ml (Eldesine, 1 mg amp., Eli Lilly GmbH, Giessen), etoposide 0.2 µg/ml (Vepesid, 100 mg amp., Bristol, Bergisch Gladbach), chlorambucil 0.06 µg/ml (Leukeran, 2 mg tablet dissolved in 1 ml abs. ethanol, Deutsche Wellcome, Burgwedel), prednisolone 250 µg/ml (Solu-Decortin, 10 mg amp., Merck, Darmstadt).

The concentrations were in accordance with those described for stem cell assays [8, 12].

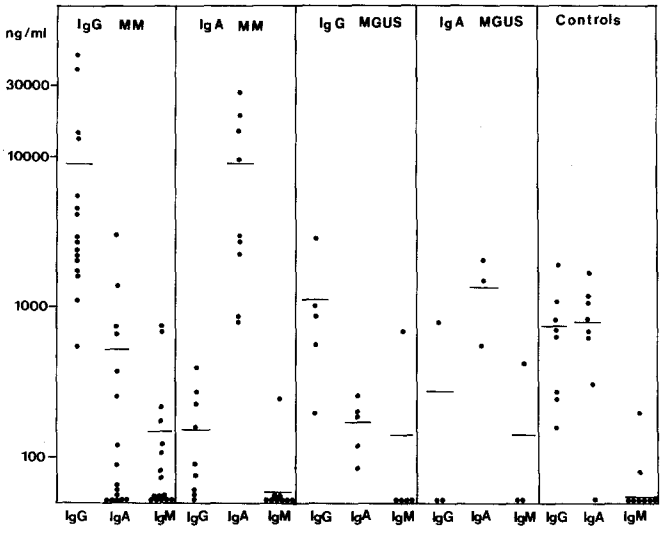

Fig. 1. Immunoglobulin production in bone marrow cultures of patients with multiple myeloma or MGUS and of control patients. Mean values are marked (———)

Enzyme-linked immunosorbent assay (ELISA). An ELISA was applied to measure mIg and β_2-microglobulin in the culture supernatants. This technique has already been described in detail elsewhere [10]. To determine mIg, rabbit anti-class specific antisera (anti-human IgG, IgA or IgM) and anti-light chain, specific antisera (anti-human kappa or lambda) were used (Dako, Boehringer, Ingelheim). Additional anti-idiotypic antisera were prepared in rabbits against the myeloma protein of patients S. J. and G. G., to allow direct measurement of the mIg in the cultures of these patients [10]. Rabbit antiserum against β_2-microglobulin was purchased from Dako, Boehringer, Ingelheim.

Microtiter plates (Nunc, Denmark) were coated with 0.05 *M* carbonate buffer, PH 9.6, containing 2 µg/ml anti-class, anti-light chain or anti-β_2-microglobulin specific antiserum for 3 h at 37 °C. Plates were washed with PBS containing 0.05% Tween 20. Culture supernatants were diluted 100–2000 times in PBS containing Tween 20, added in triplets, and incubated for 2 h at 20 °C. After washing, conjugate was added and incubated at 20 °C overnight. Conjugates between alkaline phosphatase (Sigma Chemicals) and anti-class, anti-light chain or anti-β_2-microglobulin specific antiserum were prepared as described previously [10]. The reaction between conjugated alkaline phosphatase and paraphenylphosphate was measured in a Titertek multiscan plate reader (Flow Laboratories). The measured values were transferred online to a Commodore 8032 computer system which carried out calculations on the basis of standard curves obtained with appropriate dilutions of LN protein standard serum (Behringwerke, Marburg). The monoclonal nature of supernatant Ig was ascertained by measuring the ratio of kappa to lambda determinants in culture supernatants. The ratio of kappa to lambda determinants found in the dilutions of LN protein standard serum (Behringwerke, Marburg) was defined as 1 : 1. A diluted serum from a patient with renal insufficiency served as a standard for β_2-microglobulin determinations. The amount of Ig or β_2-microglobulin produced in the cultures was defined as the difference between the total amount and the amount found in cultures containing 50 µg puromycin after the 10-day culture period.

Results

In vitro production of monoclonal and polyclonal Ig

The amounts of Ig produced in bone marrow from MM patients and controls without cytostatic drugs are shown in Fig. 1. Cultures of cells from MM patients produced large amounts of Ig of the corresponding myeloma Ig class, but only very little Ig of the other classes. In cultures of IgG-MM patients, on average 8890 ng IgG, 510 ng IgA, and

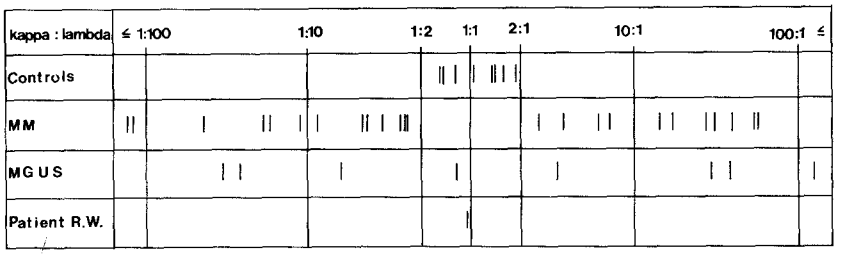

Fig. 2. Ratio of kappa-to-lambda determinants in bone marrow cultures of patients with multiple myeloma or MGUS and of control patients

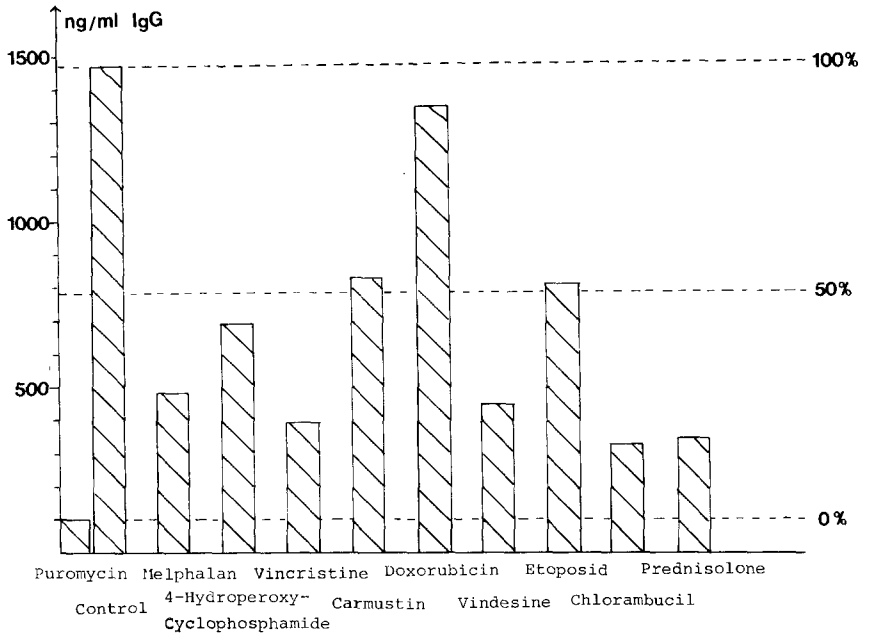

Fig. 3. Immunoglobulin found in bone marrow cultures containing no or different cytostatic drugs of an IgG myeloma patients (G. J.) who had received no prior chemotherapy

140 ng IgM were found. Cultures of IgA-MM patients produced mean amounts of 150 ng IgG, 8940 ng IgA, and 35 ng IgM. Lesser amounts of myeloma proteins were found in MGUS. In cultures of IgG MGUS patients the average contents were 1100 ng IgG, 170 ng IgA, and 140 ng IgM. In IgA MGUS patients we measured 280 ng IgG, 1330 ng IgA and 141 ng IgM. In control patients' cultures, average amounts of 800 ng IgG, and 800 ng IgA were found, while detectable amounts of IgM were only found in the cultures of two individuals. These results demonstrated that in bone marrow cultures of MM patients more than 90% of the total Ig belonged to the Ig class of the corresponding myeloma protein.

To test the monoclonal nature of the individual Ig, the kappa-to-lambda ratios were determined by an ELISA (Fig. 2). A ratio of 1 : 1 was defined by polyclonal standard Ig (LN protein standard serum, Behringwerke, Marburg). The kappa-to-lambda ratios found in the cultures of the control patients ranged from 1 : 2 to 2 : 1. With the exception of patient R. W., all ratios in cultures from MM patients were clearly outside these limits, and the Ig in the cultures could therefore be considered to be largely monoclonal. R. W. was an IgG myeloma patient whose bone marrow cultures showed 1320 ng IgG and 1520 ng IgA with a kappa-to-lambda ratio of 1 : 1.05. This Ig was therefore considered as polyclonal and the test for sensitivity to cytostatic drugs could not be performed. Bone marrow aspirate smears from patient R. W. showed no morphologic evidence of the tumor in the area where the iliac crest puncture was carried out. Except for one patient, all cul-

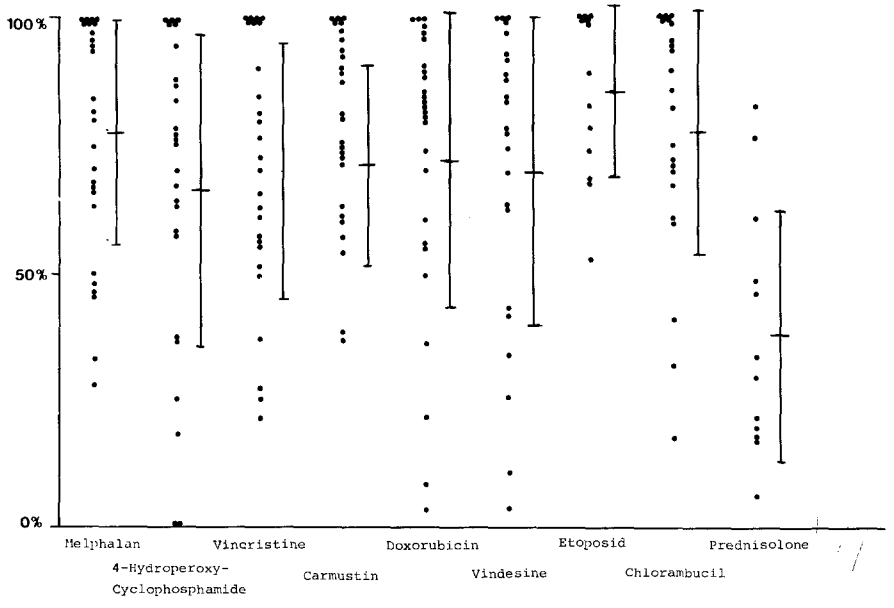

Fig. 4. mIg determined by an ELISA using the corresponding anti-class specific antiserum in cultures containing different cytostatic drugs related to cultures without drugs (100%). For each group of tests the mean value and the standard deviation are marked (±)

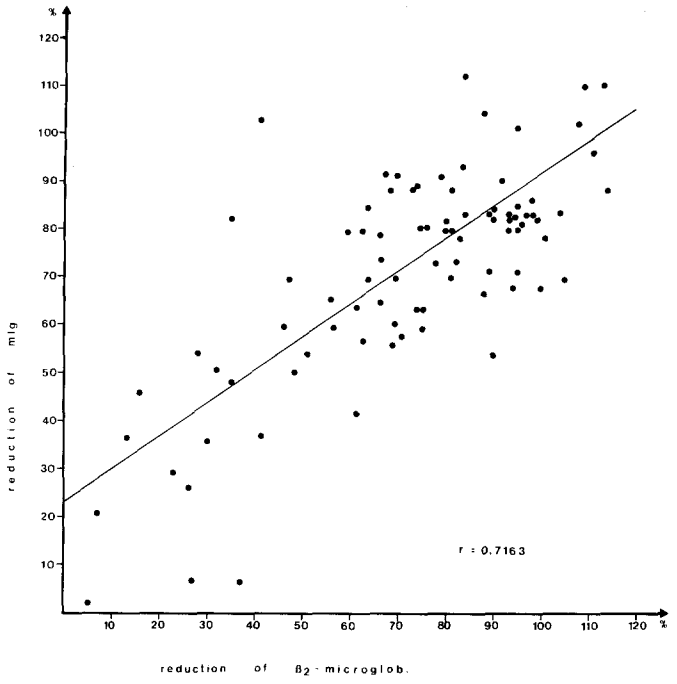

Fig. 5. Correlation of β_2-microglobulin and mIg in cultures from 16 multiple myeloma patients

tures from MGUS patients showed kappa-to-lambda ratios of the Ig similar to those for MM patients.

Demonstration of monoclonality by anti-idiotypic antisera

Anti-idiotypic antisera against the myeloma proteins of MGUS patient S. J. and MM patient G. G. were prepared, and the bone marrow culture supernatants of the corresponding patients were tested for the presence of idiotypic Ig in an ELISA. More than 90% of supernatant Ig, characterized as monoclonal by the kappa-to-lambda ratio, was also monoclonal in both cases as determined by the anti-idiotypic antisera. Other experiments using such antisera and not mitogen-stimulated cultures of 12 other patients have been done, and they have confirmed this finding [11].

In vitro cytostatic drug testing

After addition of diluted cytostatic drugs to the bone marrow cultures, we found different response patterns to the drugs regarding mIg production. An example is shown in Fig. 3. The mIg production in these cultures was strongly inhibited by chlorambucil, but hardly at all by doxorubicin. By testing the cultures of other patients we found an individual pattern of cytostatic drug sensitivity for each patient.

Table 1. Comparison of in vitro test and in vivo therapy results

Patient	Pretreated	Chemotherapy	Time of observation after in vitro testing	Clinical response	In vitro response to the drug administered in vivo (% Ig of the control culture)
I: Sensitive in vivo – sensitive in vitro					
G. E.	No	MP	8 months	CR	M (45%)
G. J.	No	MP	10 months	CR	M (28%)
L. H.	No	MP	6 months	CR	M (40%)
O. F.	No	MP	5 months	PR	M (48%)
P. H.	No	MP	8 months	PR	M (50%)
B. E.	No	MP	8 months	CR	M (33%)
L. G.	No	VCMP	11 months	CR	V (47%), C (39%), M (71%)
T. W.	Yes	VCBMP	10 months	PR	V (57%), C (0%), B (100%), M (100%)
R. N.	Yes	Vind./Dox.	6 months	PR	Vind. (62%), Dox. (49%)
II: Resistant in vivo – resistant in vitro					
G. H.	No	MP	4 months	NC	M (100%)
K. E.	No	MP	4 months	NC	M (82%)
G. G.	No	MP		P	M (85%)
P. W.	No	MP	9 months	NC	M (100%)
B. G.	No	VCMP	8 months	NC	V (80%), C (84%), M (77%)
M. B.	No	VCMP	6 months	NC	V (89%), C (91%), M (100%)
D. G.	Yes	Vind./Dox.	7 months	P	Vind. (100%), Dox. (100%)
Z. F.	Yes	Vind./Dox.		P	Vind. (100%), Dox. (60%)
III: Sensitive in vivo – resistant in vitro					
E. I.	No	MP	5 months	PR	M (100%)
D. H.	No	MP	6 months	CR	M (94%)
L. A.	No	MP	7 months	PR	M (100%)
R. K.	No	MP	6 months	PR	M (94%)
IV: Resistant in vivo – sensitive in vitro					
None					

Table 2. Test results for two patients tested on two occasions (mIg as % of that in the control culture)

	Patient B. G. VCMP therapy		Patient T. W. VCBMP therapy	
	Before	After 8 months	Before	After 10 months
Control	100%	100%	100%	100%
Melphalan	77%	60%	100%	73%
4-Hydroperoxy cyclophosphamide	84%	58%	0%	75%
Vincristine	80%	63%	57%	70%
Carmustine	81%	74%	100%	71%
Doxorubicin	85%	70%	100%	80%
Vindesine	77%	63%	n.d.	89%
Etoposid	n.d.	78%	n.d.	89%
Chlorambucil	75%	60%	n.d.	70%
Prednisolone	n.d.[a]	21%	n.d.	16%

[a] n.d., not done

A summary of these results is given in Fig. 4. Prednisolone induced average mIg production that was 38% of that in the control cultures. An average reduction to between 66% and 85% of the control values was achieved with the other different cytostatic drugs. Whereas with 4-hydroperoxy cyclophosphamide, doxorubicin and vindesine the response varied considerably between individual patients, resulting in a high standard deviation for the total group, a more even reduction was observed following addition of melphalan or carmustine. Whether these results indicate a better descrimination between sensitivity and resistance for the former group of drugs or are simply a chance observation in this group of patients remains to be established by further experience with a larger number of patients.

As a second marker we measured β_2-microglobulin in the culture supernatants. The response of this marker to the cytostatic drugs correlated well with the response of the mIg. The results for 16 patients are shown in Fig. 5.

Clinical evaluation of test results

To compare the test results with the in vivo response of the patients, the following definitions were adopted:

A. A patient was considered sensitive in vivo if the serum myeloma protein content was reduced by 25% or more after treatment with a standard cytostatic drug combination (PR and CR).
B. A patient was considered sensitive in vitro if the mIg produced in vitro by myeloma bone marrow cultures was reduced by 50% or more after addition of a cytostatic drug.
C. A patient sensitive in vivo to a cytostatic drug combination was termed sensitive in vitro to that combination if the bone marrow cultures also reacted to one or more drugs of the combination according to the criteria given under A and B.

In vitro test results obtained with prednisolone were not taken into account, because too few patients were tested (Fig. 5) and the few results obtained obviously did not reflect the in vivo situation if 50% reduction of the mIg production in vitro represented sensitivity.

These definitions were used in the evaluation of 24 MM patients. Data from 3 patients had to be omitted: 1 who had not been observed for an adequate period of time; 1 who was not treated with the drugs being tested; and patient R. W. (see above). The results recorded in the remaining 21 MM patients are shown in Table 1. Nine patients were sensitive both in vivo and in vitro, while eight were resistant both in vivo and in vitro. Discrepant results were obtained in four patients, who although sensitive in vivo had been classified as resistant in vitro. All four of these patients were treated with the drug combination of melphalan and prednisolone. For the 21 MM patients, the test system had a predictive value of more than 80%. Two patients have so far been tested twice (Table 2). Patient B. G. had almost the same profile of sensitivity to the drugs after 8 months' VCMP therapy. In contrast, the tumor of patient T. W. had almost completely lost its sensitivity to 4-hydroperoxy cyclophosphamide after 10 months of VCMP therapy.

Discussion

Individual tumor chemotherapy instead of standard protocol treatment would be of obvious benefit to the patient, since the administration of ineffective but nevertheless toxic drugs could be avoided. This paper describes the development and application of a new test method which may advance individual chemotherapy in multiple myeloma. For this purpose, a number of stem cell assays which measure the inhibition of colony formation by cytostatic drugs have been developed, which are described elsewhere [5, 7, 8, 12]. Because of the considerable technical outlay these tests involve, they are difficult to perform in a large number of patients. Our test estimated the growth inhibition of the tumor cells indirectly by determining the mIg tumor product in bone marrow cell cultures with an ELISA. Since direct proof of monoclonality by the use of anti-idiotypic antibodies is impossible in a test where results are to be obtained within days, we successfully tried to estimate the mIg production in bone marrow cultures with an ELISA, using anti-class specific antisera for quantitation, and anti-light chain specific antisera for determination of the abnormal kappa-to-lambda ratios characteristic for mIg production. Nearly all Ig found in bone marrow culture supernatants of MM patients with disseminated disease was indeed found to be monoclonal (Figs. 1 and 2), in contrast to that in cultures of peripheral blood mononuclear cells in MM patients, where in vitro Ig production of cells belonging to the tumor clone was not consistently observed and more polyclonal Ig was produced [10]. The monoclonal character of the Ig produced in myeloma bone marrow cultures was again confirmed in this work by using individual anti-idiotypic antibodies in two patients. Identical results were obtained by the combined use of anti-Ig class and anti-light chain (kappa, lambda) antisera.

As a second marker for the myeloma tumor cell proliferation in vitro we measured β_2-microglobulin produced in the bone marrow cultures of the patients. β_2-Microglobulin is the light-chain protein of the class I histocompatibility membrane marker, which can be found in all nucleated cells, and is also produced by MM cells, where it reflects the tumor proliferation and mass [1, 4]. In bone marrow culture supernatants of the patients we tested the concentrations of β_2-microglobulin correlated well with reduction

of mIg production induced by the cytostatic drugs (Fig. 5). This finding bears out the potential of this test system for application to other disseminated bone marrow tumors or other tumors, providing short term cultures can be established, which is currently evaluated in our laboratory.

To correlate the in vitro responses to the in vivo reaction of the patients' tumors we had to formulate some definitions, which may be subject to review as more experience is accumulated. Thus, possible additive effects of cytostatic drugs in combinations were neglected, and the role of steroids for tumor inhibition was not considered ready for evaluation on the basis of the available data. With these premises, the predictive value of more than 80% for the 21 test patients is acceptable and comparable with other published drug sensitivity tests for MM patients. In one patient a change in drug sensitivity was observed upon repeated in vitro testing, so that drug sensitivity monitoring may be developed as a second application of this method. False drug resistance was indicated in four patients subsequently successfully treated with melphalan and prednisolone; melphalan should possibly be used at higher concentrations in the test for better discrimination between sensitivity and resistance of individual tumors.

In vitro cytostatic drug sensitivity tests have to be critically and carefully evaluated for their usefulness in clinical practice. This was recently discussed for stem cell assays by Selby et al [14] and Von Hoff [15]. The test system described in this paper is easy to perform, so that regular cytostatic drug testing in MM patients can be performed along with the cytological evaluation of bone marrow samples obtained before and during treatment. Therefore, it is possible to test this system in a large number of patients before using it for practical purposes. Studies including evaluation of cell numbers, proliferation rates, and cell morphology during culture time, and correlation of this assay with others are currently in progress and will allow a more complete judgement as to the clinical value of this in vitro sensitivity test system.

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