Risk Factors Associated With β-Amyloid$_{(1-42)}$ Immunotherapy in Preimmunization Gene Expression Patterns of Blood Cells

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Background: A phase 2a, double-blind, placebo-controlled, multicenter study was conducted to evaluate safety, tolerability, and pilot efficacy of immunization with β-amyloid$_{(1-42)}$ in patients with Alzheimer disease. Six immunizations were planned but were halted when meningoencephalitis was recognized as an adverse event in 6% of immunized patients.

Objective: To identify biomarkers associated with both the risk of meningoencephalitis and antibody responsiveness.

Participants: One hundred fifty-three patients with mild to moderate Alzheimer disease.

Main Outcome Measure: Association between response to immunization and preimmunization expression levels of 8239 messenger RNA transcripts expressed in peripheral blood mononuclear cells that had been collected at the screening visit.

Results: Expression patterns of genes related to apoptosis and proinflammatory pathways (tumor necrosis factor pathway in particular) were identified as biomarkers of risk for the development of meningoencephalitis. Expression patterns of genes related to protein synthesis, protein trafficking, DNA recombination, DNA repair, and cell cycle were strongly associated with IgG response to immunization.

Conclusions: Candidate biomarkers associated with risk of immunotherapy-related meningoencephalitis were detected in blood collected prior to treatment. In addition, a different set of biomarkers were identified that were associated with the desired outcome of IgG response.

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ALZHEIMER DISEASE (AD) IS characterized histopathologically by the accumulation of amyloid plaques and neurofibrillary tangles and by increased rates of neuronal atrophy. The recognition of the β-amyloid$_{(1-42)}$ (Aβ$_{[1-42]}$) section of amyloid as a major component of AD-related plaques[1] led to an experimental therapeutic strategy against AD based on clearance of plaque by antibodies directed against Aβ$_{(1-42)}$.[2] Studies in transgenic mouse models of cognitive impairment and amyloid plaque–associated central nervous system pathologic features demonstrated that immunization with AN1792, a peptide immunogen consisting of Aβ$_{(1-42)}$, resulted in improved cognitive function and inhibited the development of AD-like amyloid plaques, neuritic dystrophy, and gliosis in mice.[3-8] Following a phase 1 study,[9] a phase 2a, double-blind, placebo-controlled, multicenter study was conducted to evaluate safety, tolerability, and pilot efficacy of AN1792 (Aβ$_{[1-42]}$) administered with QS-21 adjuvant in 372 patients with mild to moderate AD.[10-12] Six immunizations were planned but were halted when meningoencephalitis was recognized as an adverse event associated with AN1792 immunization. At the time that

For editorial comment see page 1506

treatments were discontinued, the maximum number of immunizations received was 3 (by 24 patients), with the majority of patients (274) having received 2 immunizations. Ultimately, 18 of 300 immunized patients developed meningoencephalitis.[10] Cognitive function, anti-AN1792 antibody, cerebrospinal fluid tau, and cerebrospinal fluid Aβ$_{(1-42)}$ were assessed un-

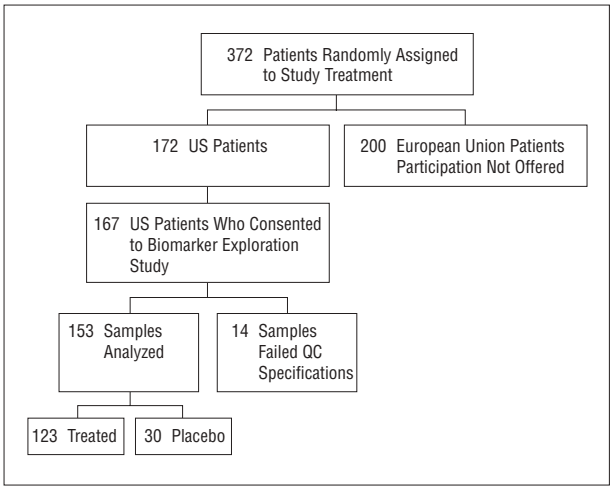

Figure 1. Participants in exploratory biomarker study. QC indicates quality control.

til the conclusion of the original follow-up period. Antibody responders were compared with placebo controls. Two tests, levels of cerebrospinal fluid tau and a neuropsychological test battery, gave results favoring patients with a positive IgG titer.[12] To investigate whether candidate biomarkers of response to immunotherapy could be identified, we used the Affymetrix GeneChip microarray technology (Affymetrix, Santa Clara, Calif) to conduct an exploratory pharmacogenomic study of the preimmunization gene expression patterns of the peripheral blood mononuclear cells of enrolled patients.

METHODS

PARTICIPANTS

A total of 372 patients with mild to moderate AD, 172 from the United States and 200 from the European Union, were enrolled in the clinical trial. Participation in the exploratory biomarker portion of the study was optional and offered only to patients enrolled in the United States. Informed consent was obtained after approval by local institutional review boards. **Figure 1** shows the total number of patients in the clinical study and the number who consented to inclusion.

ANALYTICAL SAMPLE AND GENE EXPRESSION ASSAY

The analytical sample consisted of peripheral blood mononuclear cells collected prior to immunization. Blood was drawn at the screening visit (9-54 days prior to first immunization) and shipped overnight to the Wyeth Clinical Pharmacogenomic Laboratory in Andover, Mass, and the peripheral blood mononuclear cells were purified as previously described.[13] RNA was purified (from 2×10^6 cells) using QIA shredders (Qiagen, Santa Clarita, Calif) and Qiagen Rneasy mini-kits. Labeled targets for oligonucleotide arrays were prepared using 50 ng of total RNA. Biotinylation of complementary RNA (generated using 2-cycle in vitro transcription amplification) was hybridized to the HG-U133A Affymetrix GeneChip Array, and gene expression levels were measured by conversion of signal values to normalized scaled frequency in parts per million.[14] Data for 9678 probe sets that were called "present" and with a frequency of 10 ppm or more in at least 1 of the samples were subjected to the statistical analyses described in the next subsection, while probe sets that did not meet these criteria were excluded. The search for gene expression levels associated with response to treatment was conducted by comparing preimmunization expression levels between subjects grouped according to postimmunization response. The postimmunization response groups analyzed are shown in **Table 1**.

Table 1. Study Samples

Patients	Total	Male	Female
No. of treated patients	**123**	66	57
Treated IgG responders	**25**	12	13
Treated IgG partial responders	**28**	12	16
Treated IgG nonresponders	**70**	42	28
Treated IgM responders	**40**	20	20
Treated IgM partial responders	**39**	18	21
Treated IgM nonresponders	**44**	28	16
Patients with meningoencephalitis	**5**	0	5
No. of placebo patients	**30**	12	18

STATISTICAL ANALYSES

The clinical and gene expression databases were merged using SAS (SAS Institute Inc, Cary, NC), and SAS was used for all analyses unless otherwise noted. Analyses were conducted to identify factors that might have confounding effects on associations between gene expression levels and response group. Preimmunization differential blood cell counts and sex were 2 such factors investigated, and both were identified as significant covariates. For each gene, analysis of covariance was used to test for associations of expression level with these 2 covariates. To avoid potential confounding with IgG response or the development of meningoencephalitis, these analyses of covariance were run using data only from IgG nonresponders (n=70), none of whom developed meningoencephalitis. Log-transformed expression was modeled as a function of sex and the monocyte-lymphocyte ratio. Genes were considered significantly associated with either sex or the monocyte-lymphocyte ratio if the unadjusted F test *P* value for the respective effect was $<.01$. Sequences significantly associated with monocyte proportion and/or sex were removed from further analysis, leaving 8239 sequences subject to further analyses.

The binary logistic regression model was used to determine if significant associations existed between preimmunization gene expression levels and postimmunization development of meningoencephalitis. Raw *P* values were adjusted for multiplicity according to the false discovery rate (FDR) procedure.[15,16] Treated patients who developed meningoencephalitis (n=5) were compared with those who did not (n=118). The small number of patients with meningoencephalitis resulted in large odds ratios with some exceedingly wide confidence intervals (2-3 orders of magnitude). Genes were selected as significantly associated with meningoencephalitis if: (1) the odds ratio between patients with and without meningoencephalitis was more than 3-fold; (2) the FDR was less than 0.1 (a criterion that allows for an estimated 10% false-positive identifications); (3) the odds ratio for association with meningoencephalitis was at least 2 times greater than that for association with IgG response; (4) the FDR for association with IgG response was greater than 0.1; and (5) the odds ratio for IgG response was less than 2-fold. Because of the observation that some genes with IgG odds ratios between 2- and 4-fold had meningoencephalitis odds

ratios up to hundreds-fold higher, exceptions to the last criterion were made when the odds ratio for association with meningoencephalitis was at least 5-fold greater than that for association with IgG response.

The ability of 2-gene models to discriminate between patients with and without meningoencephalitis was evaluated by logistic regression models using as covariates all 287 661 pairwise combinations of genes meeting the criteria for association with meningoencephalitis. For each model, the sum of the absolute values of the log odds for all subjects was used as a ranking measure to indicate the strength of the discrimination. To estimate the FDRs for this large set of logistic regression models, the full analysis was rerun 200 times with random permutation of the class labels to compute resampling-based FDRs.[17] These analyses were carried out using R statistics package 1.9.1.[18]

Antibody response groups were defined prior to unblinding, with group assignments based on postimmunization maximum titer. For both IgM and IgG, the groups were: (1) nonresponders (titer <200 mg/dL); (2) partial responders (200 mg/dL ≤titer <2200 mg/dL); and (3) responders (titer ≥2200 mg/dL). The proportional odds logistic regression model was used to determine if significant associations existed between preimmunization gene expression levels and postimmunization response groups. The analyses were run both using all immunized subjects in the study (n=123) and with the exclusion of the 5 patients with meningoencephalitis (n=118). (All US patients with meningoencephalitis were IgG responders, and all but 1 European Union patient with meningoencephalitis were IgG responders.) Genes were selected as significantly associated with response if: (1) the FDR for association with response was less than 0.1; (2) the odds ratio between responders and others (nonresponders plus partial responders) was greater than 3-fold; (3) the FDR from the analysis excluding patients with meningoencephalitis was less than half the FDR for association with meningoencephalitis; and (4) the FDR for association with meningoencephalitis was greater than 0.1.

RESULTS

The selection criteria for association with meningoencephalitis were met by a large number of sequences (from 689 genes and 8 unmapped sequences), signifying a robust association between the preimmunization gene expression profile and the postimmunization development of meningoencephalitis. Among meningoencephalitis-associated genes were many well-established to mediate proinflammatory processes. To track the biological pathways and functional networks implicated in the development of meningoencephalitis by the association with this large gene set, the 689 genes were analyzed using Ingenuity Pathway Analysis (Summer '04 Release V1)[19] to reveal relationships between them. The pathway analysis application assigned 56% of the 690 genes to High-Level Functions and Global Canonical Pathways. Significantly represented were genes related to the control of cell death (apoptosis) and proinflammatory immune response or to the downstream functions of control of cell proliferation and protein synthesis. For example, Ingenuity Pathway Analysis reports *P* values as ranging from 7.46E-7 to 4.65E-2 for the significance of the link between meningoencephalitis-associated genes and cell-death categories. (*P* value in this context is a measure for how likely it is that genes associated with the risk of meningoencephalitis participate in cell death. The range of *P* values reflects the significance at different levels of different levels of cell-death pathways). Genes related to tumor necrosis factor/Fas, transforming growth factor-β, and p53 pathways, central pathways in the control of the immune system, were highly represented among genes related to the control of cell death. A selection of tumor necrosis factor–associated genes and the strength of their association with meningoencephalitis is presented in **Table 2**.

Table 2. Selection of Genes Associated With Both Meningoencephalitis and TNF, a Proinflammatory Pathway Involved in Cell Death

Gene Name	Odds Ratio for Association With Meningoencephalitis	FDR for Association With Meningoencephalitis	Associated Cell Death Pathways
STAT1	230.42	0.004	TNF, p53, TCR
STAT5A	56.76	0.065	TNF
CSE1L	38.75	0.019	TNF
TRIAD3	29.38	0.039	TNF
PDCD11	15.89	0.036	TNF
IL19	8.87	0.031	TNF
STAT3	6.61	0.011	TNF, p53, TGF
MMP7	6.51	0.044	TNF
NFKB1	5.35	0.066	TNF
TNFSF10	4.81	0.042	TNF
IL12B	4.43	0.067	TNF, TGF
KRAS2	3.71	0.050	TNF
TNF	3.47	0.067	TNF
TNFRSF9	3.45	0.060	TNF
LRDD	0.22	0.049	TNF, p53
ZFP36	0.11	0.029	TNF
PTEN	0.11	0.048	TNF, p53
PIK3CA	0.08	0.056	TNF, TGF
TRAF6	0.05	0.062	TNF
PRKRA	0.03	0.022	TNF

Abbreviations: FDR, false discovery rate; TCR, T-cell receptor; TGF, transforming growth factor; TNF, tumor necrosis factor.

Many of the meningoencephalitis associations identified were so significant that they exceeded by orders of magnitude the selection criteria. For example, the gene most significantly associated (FDR=0.004; unadjusted *P* value=5.07E-7; odds ratio, 230) was *STAT1*, a critical gene in a proinflammatory signal transduction pathway. Patients with high levels of *STAT1* in peripheral blood mononuclear cells prior to immunization had an extremely elevated risk of developing postimmunization meningoencephalitis. Low expression levels of *HEAB* were strongly associated with risk of meningoencephalitis (odds ratio, 1.0E-4). For 364 of the meningoencephalitis-associated sequences, the odds ratios (for elevated risk) were greater than 10-fold (greater than 10 or less than 0.1). Data for all meningoencephalitis-associated sequences are available on request.

A pairwise combination logistic regression approach was designed to find the 2-gene combination that best distinguished all patients with meningoencephalitis from those without. For 18 of the top 20 2-gene combinations, one of the genes in the 2-gene combination was either NPukP68 or *STAT1*, indicating that these 2 genes

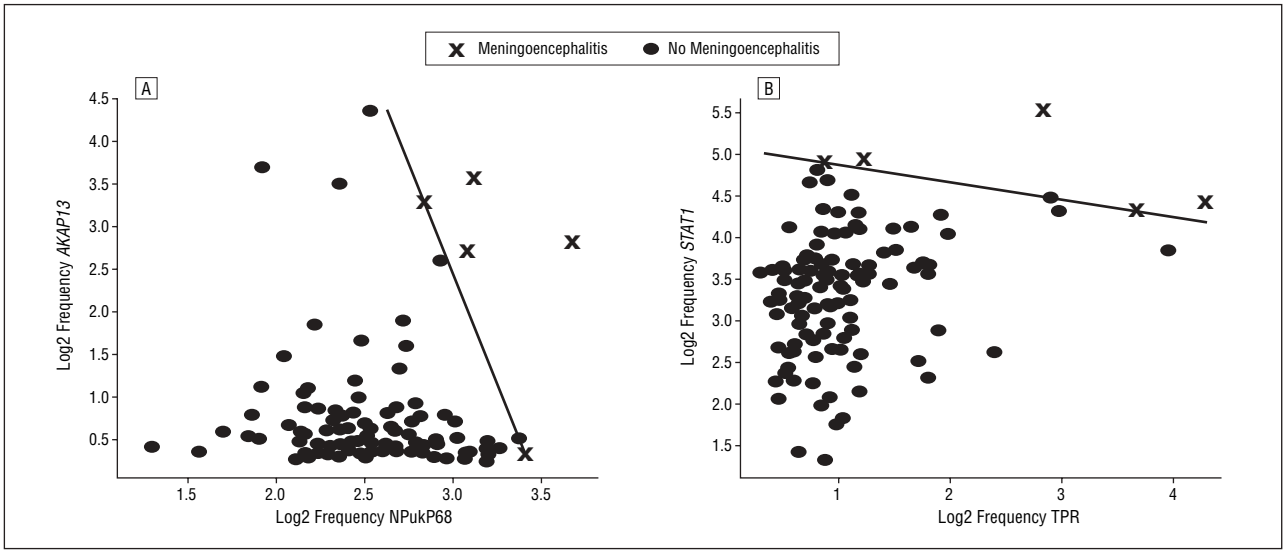

Figure 2. Optimal biomarkers selected on the basis of distinguishing all patients with meningoencephalitis. A, The expression levels of each patient for the top-ranked 2-gene combination. B, The expression levels for the top-ranked 2-gene combination containing *STAT1* (ranked third overall). TPR indicates translocated promoter region (to activated MET oncogene).

are the best candidate biomarkers of risk. **Figure 2**A shows the expression levels of each patient for the top-ranked 2-gene combination, and Figure 2B shows the expression levels for the top-ranked 2-gene combination containing *STAT1* (ranked third overall).

No genes met the criteria for significant association of preimmunization gene expression levels and postimmunization IgM titer. In contrast, there were 366 sequences (from 318 genes and 17 unmapped sequences) that met the selection criteria for association with IgG response. Ingenuity Pathway Analyses indicate that, prior to immunization, the ability to mount an IgG response is highly correlated with expression patterns of genes directly involved in protein synthesis. The *P* value for the link between this biological function and the ability to mount an IgG response ranges from 9.53E-12 to 1.29E-3. We identified an additional 22 sequences that directly participate in translational events, signifying that the link is even more significant than reported by Ingenuity. Remarkably, all of the genes associated with IgG response and directly involved in the protein synthetic machinery were expressed at higher levels in IgG responders. In contrast, almost half (42%) of the IgG response–associated genes involved in other functions were expressed at lower levels in IgG responders. Functions significantly represented among these genes were transcription, cell cycle, cell growth and proliferation, protein trafficking, DNA repair and recombination, and protein synthesis regulation. The selection of the most significant of these genes is presented in **Table 3**. As was also true for the list of genes associated with meningoencephalitis, for many genes associated with IgG response, the significance of the association (as measured by FDR) exceeded the selection criteria by orders of magnitude with the most significant FDR observed = 0.0003 (*P* value unadjusted for multiplicity = 1.07E-7).

The FDRs and odds ratios for all 366 genes associated with IgG response are available on request.

COMMENT

We conducted a prospective search for biomarkers associated with responsiveness to $A\beta_{(1\text{-}42)}$ immunotherapy using as the analytical sample RNA purified from the blood of 153 patients who had donated samples for this search at the screening visit. The initial objective was to identify biomarkers that might aid in the preidentification of patients most likely to mount antibody responses in any subsequent studies. On the development of treatment-related meningoencephalitis,[10] the gene expression profiles of 118 treated meningoencephalitis nondevelopers were compared with those of 5 meningoencephalitis developers. Highly significant differences were identified, and these differences constitute a list of candidate biomarkers for identifying those most at risk for immunotherapy involving active immunization with $A\beta_{(1\text{-}42)}$. Since the biomarkers reported herein were identified using gene expression data from all 5 patients with meningoencephalitis and have not been confirmed in an independent study, they must be regarded as candidate or potential biomarkers. The potential utility of biomarkers that, prior to the initiation of treatment, could identify those most at risk is underscored by data indicating some treatment-related cognitive improvement in IgG titer–positive patients.[12] The cognitive benefit was observed despite the suspension of treatment at a time when no patient had received more than 3 of the 6 planned immunizations.

Functional annotation of genes associated with meningoencephalitis indicates a preponderance of genes in pathways critical to the control of the immune system in particular. Those who developed meningoencephalitis had, prior to immunization, detectable perturbations in pathways controlling the tumor necrosis factor and other proinflammatory and apoptotic cascades. Perturbations favoring both antiapoptotic and proapoptotic activities were detected, suggesting perhaps compensatory activation to counteract deleterious effects of perturbation in apoptosis. This is also supported by perturbations in a large num-

Table 3. A Selection of Genes Showing Significant Association Between Preimmunization Gene Expression Levels and Postimmunization IgG Titer

Ingenuity Category	Gene	FDR	Odds Ratio	Gene Description
Protein synthesis	*RPL26*	0.0012	3.342	Ribosomal protein L26
DNA repair, replication, recombination	*XRCC2*	0.0017	3.033	X-ray repair complementing defective repair in Chinese hamster cells 2, role in VDJ recombination
Protein synthesis	*PTBP1*	0.002	0.112	Polypyrimidine tract binding protein
Protein synthesis	*MAP2K3*	0.0028	0.195	Mitogen-activated protein kinase kinase 3
Protein trafficking	*XPO7*	0.0029	0.298	Exportin 7
Protein synthesis	*RPL4*	0.0029	6.268	Ribosomal protein L4
Protein synthesis	*RPL7*	0.0034	6.044	Ribosomal protein L7
Protein synthesis	*RPL14*	0.0037	5.31	Ribosomal protein L14
DNA repair, replication, recombination	*CDKN1A*	0.004	0.325	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
DNA repair, replication, recombination	*CDK2*	0.004	4.295	Cyclin-dependent kinase 2
DNA repair, replication, recombination	*PAFAH1B1*	0.0045	0.212	Platelet-activating factor acetylhydrolase β subunit
Protein synthesis	*PABPC4*	0.0046	0.256	Poly(A) binding protein, cytoplasmic 4 (inducible form)
Protein trafficking	*ARF3*	0.0048	0.32	ADP-ribosylation factor 3
Protein synthesis	*RPL6*	0.0055	8.309	Ribosomal protein L6
DNA repair, replication, recombination	*PRKDC*	0.0063	3.401	Protein kinase, DNA-activated, catalytic polypeptide, role in VDJ recombination
Protein synthesis	*RELA*	0.007	0.26	V-rel reticuloendotheliosis viral oncogene homologue A, nuclear factor of κ light polypeptide gene enhancer in B cells 3, p65 (avian)
Protein synthesis	*SSB*	0.008	3.664	Sjögren syndrome antigen B
Protein trafficking	*MCM3AP*	0.0092	3.627	MCM3 minichromosome maintenance deficient 3 associated protein

Abbreviations: ADP, adenosine diphosphate; FDR, false discovery rate; VDJ, variable diversity joining.

ber of cell-cycle, growth, and proliferation genes. The *STAT* gene family plays central roles in proinflammatory cytokine activation and in apoptotic cascades. Perturbation in the expression levels of *STAT1*, *STAT3* (3′ untranslated region), and *STAT5* was found to be a highly significant risk factor for meningoencephalitis. High expression of a variety of other genes involved in proinflammatory cascades, such as IL-9, IL-19, IL-25, IL-27R, and CD80, was also associated with meningoencephalitis. Elevated expression of the coding region and decreased expression of the 3′ untranslated region of *STAT5B* were associated with development of meningoencephalitis, suggesting that variants of *STAT5B* messenger RNA make different contributions to the "meningoencephalitis-prone" gene expression pattern. Despite these perturbations in proinflammatory pathways, no preimmunization clinical symptoms have been identified that distinguished patients who developed meningoencephalitis. The data presented in this report therefore suggest that both proinflammatory and compensatory pathways were in functional balance but, for this small minority of patients, immunization with $A\beta_{(1-42)}$ disturbed that balance.

In addition to meningoencephalitis-associated candidate biomarkers, we also report on the identification of biomarkers related to antibody responsiveness. These biomarkers of risk of nonresponsiveness were sought because of the relatively low incidence (48%) of responsiveness observed in the phase 1 study.[9] No associations that met the prespecified criteria were identified between IgM response and preimmune gene expression levels. However, the expression levels of 318 genes differed between IgG responders and nonresponders. Expression levels in partial responders (200 mg/dL ≤titer <2200 mg/dL) were consistently intermediate between nonresponders (titer <200 mg/dL) and responders (titer ≥2200 mg/dL), a trend that provides additional evidence of the relationship between preimmunization gene expression pattern and IgG response.

In marked contrast to the meningoencephalitis-associated biomarkers, the vast majority of genes associated with IgG response are related to biological functions that are not specific to the immune system. Rather, the functions most significantly associated with IgG responsiveness—protein synthesis and trafficking, RNA processing, and cellular assembly and organization—are required for all biological functions. These findings raise the possibility that antibody nonresponsiveness in this study was related to a generalized decline, and since the participants were elderly, the role of age is an obvious factor to consider. The incidence of responsiveness to immunization is known to decline with age.[20-24] Therefore, the relatively low incidence of responsiveness in this study may be related to age, which, the data reported herein suggest, may in turn be related to a decline in the robustness of biological systems in general. We are currently investigating the influence of age on the expression levels of genes directly involved in protein synthesis and the other functions identified in this study as associated with IgG response.

We have shown that candidate biomarkers associated with response to $A\beta_{(1-42)}$-based immunotherapy are detectable in blood prior to initiation of therapy. It would

be of clear utility to the AD research community if these findings can be converted to a validated test for preidentification both of those most at risk and those most likely to give a favorable response.

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